

# Biofilm formation in *Bacillus thuringiensis*

Investigation of the roles of a putative cell surface adhesin  
and a chemotaxis-related protein responsive to cyclic-di-  
GMP

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## Abstract

The *Bacillus cereus* group of bacteria is a subgroup in the *Bacillus* genus and consists of six different species. Research has evolved mainly around *B. cereus*, *B. thuringiensis* and *B. anthracis*, an opportunistic pathogen capable of food poisoning and infections in mammals, an insecticidal pathogen which can also be an opportunistic pathogen in mammals, and an opportunistic pathogen capable of causing cutaneous and or/systemic anthrax in mammals, respectively. These species are closely related based on analysis of chromosomal markers, but pathogenicity patterns vary mainly due to virulence factors carried on plasmids. Taxonomic differentiation has been, and continues to be, difficult, as many features used to classify the organisms are plasmid-borne.

Several studies have shown that many strains in the *B. cereus* group are capable of producing biofilms, multicellular aggregates of cells that may contribute to virulence, antibiotic resistance and to reservoirs forming in food production facilities and hospitals. *B. cereus*, ubiquitously found as spores in soil, has been implicated in several cases of food spoilage. Research of biofilm formation in *B. cereus* is, therefore, important for economic and health-related purposes.

One research group reported in 2008 that two c-di-GMP responsive riboswitches, non-translated regions of mRNA located at the 5'-end that may govern further expression of a downstream gene, had been identified in *Bacillus cereus* strains ATCC 14579 and ATCC 10987. Sequence searches based on the sequences of these riboswitches showed that two similar riboswitches were present in *B. thuringiensis* 407, and two genes were located downstream of these riboswitches, whose proteins contained domains typical of cell surface adhesion (Bt407\_1060) and chemotaxis (Bt407\_0422) proteins, respectively. As c-di-GMP is an important regulator of biofilm formation in gram-negative bacteria, it was decided to proceed with functional studies of these two proteins. The aim of this thesis was to expand on initial research performed on this topic in the group.

The functional studies performed in this thesis confirmed that a gene deletion in Bt407\_1060 (cell surface adhesion protein) resulted in reduced biofilm formation in a microtiter plate screening assay. Previous studies on adhesion proteins in gram-positive bacteria have shown

an effect on biofilm formation when genes encoding the adhesion proteins were removed. No effect was observed on biofilm formation in a screening of the Bt407\_0422 (methyl-accepting chemotaxis protein) gene deletion mutant compared to wildtype. Previous research in other bacteria has deemed chemotaxis as non-essential to biofilm formation, as opposed to the presence of flagella or motile behaviour, which seems of importance. However, in order to ratify these results, complementation and overexpression of the genes would be necessary. Future research should also focus on the role of c-di-GMP in the regulation of biofilm formation by *B. cereus* group bacteria to enhance our understanding of c-di-GMP regulated processes in gram-positive bacteria.

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# Chapter 1: Introduction

## 1.1 The *Bacillus* genus

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The *Bacillus* genus is in the family *Bacillaceae* and consists of rod-shaped, gram-positive, aerobe/facultative anaerobic, spore-forming bacteria. The natural habitat ranges from soil and vegetation to insect intestines, and some species are pathogenic to insects and/or animals/mammals while most are considered non- pathogenic.

The vegetative cells range from 0.5 by 1.2 to 2.5 by 10  $\mu\text{m}$  in diameter and can grow at optimal temperatures ranging from 25 to 37°C, although thermophilic and psychrophilic members are capable of growth at temperatures as high as 75°C or as low as 3°C [6]. The members of the genus may grow at a variety of pH values ranging from 2 to 10 [6]. The G+C content of the DNA of species within the genus can vary from 32 to 69%. Most strains are catalase positive, possess peritrichous flagella, and sporulate in air, differentiating members of this genus from the clostridia [6].

### 1.1.1 *Bacillus cereus* group

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The *B. cereus* group is a subgroup within the *Bacillus* genus, and consists of several species, all which are closely related and some share very similar chromosomal content. The species included in the group are *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*. The first three species are considered opportunistic pathogens in mammals and insects while the last three species are generally considered non- pathogenic [7]. In addition, a strain in the *B. cereus* group, *Bacillus cereus* *subsp. cytotoxis*, is also important, as it has been implicated in severe cases of food

poisoning leading to human death, and belongs to a phylogenetically remote subgroup of thermotolerant isolates [8].

Based on chromosomal phylogeny, the *B. cereus* group has been suggested to comprise variations of the same species, but with distinct phenotypic differences. Some scientists have suggested that they are all members of a single species, *B. cereus sensu lato* [9].

### 1.1.2 Genomics of the *Bacillus cereus* group

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It has been difficult to establish a commonly accepted taxonomic classification between the species in the *Bacillus cereus* group. Several techniques, including PCR patterns [10], multi-locus enzyme electrophoresis [11], ribotyping [12] and carbohydrate profiles [13] have been employed in order to try to differentiate between different species in the *B. cereus* group. Additionally, DNA–DNA hybridization and comparison of 16S or 23S rRNA sequences or 16S–23S rRNA [9], have also been used.

Phenotypic traits have been used to distinguish *B. anthracis* from other *Bacillus* species in the lab due to a nonsense point mutation in the global transcriptional translator PlcR which results in no or severely reduced activity of PlcR- regulated genes within the bacteria [14]. These difference include being non-motile, non-hemolytic, negative for phospholipase C (PLC) production, and sensitive to  $\gamma$  phage [7].

Genes located on plasmids account for much of the differences in pathogenicity observed between *B. cereus*, *B. anthracis* and *B. thuringiensis*. A strain designated as *B. cereus* (*Bacillus cereus* G9241) has been found to contain two plasmids, one plasmid which closely resembles the pXO1 plasmid found in *B. anthracis* and a second plasmid carrying genes coding for a polysaccharide capsule. This particular strain caused a disease resembling anthrax in American patients [15]. *B. cereus* strains isolated from dead chimpanzees in Africa have been shown to contain plasmids which closely resemble pXO1 and pXO2 [7, 16]. These cases where the plasmids have been shown to be able to transfer between

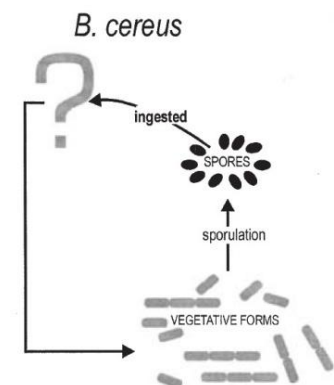
different cells and strains have made characterization by plasmid content non- satisfactory. *B. thuringiensis* strains cured of their plasmids are indistinguishable from *B. cereus*. Several researchers have concluded that these three species share the same ancestor, and has successfully developed into an individual lineage.

## 1.2 Strains of the *Bacillus cereus* group

### 1.2.1 *Bacillus cereus*

*Bacillus cereus* is considered an opportunistic pathogen in animals and mammals. Most *B. cereus* strains are motile (flagella), form wide, opaque, irregular colonies when grown on solid surface mediums and utilize glucose as the main carbon source [17].

The *B. cereus* endosymbiotic life cycle consist of three distinct phases. *B. cereus* is ubiquitously found as spores in soil, but spores are able to germinate and colonize the gastro-intestinal system in mammals and in insects [5] and other organic matter .Vegetative cells sporulate upon depletion of nutrients and other environmental signals and return to the soil. There has been dispute whether the life cycle in *B. cereus* is pathogenic or not [5].



**Figure 1: Life cycle of *Bacillus cereus* (figure from Jensen et. al [5])**

Alternatively, *B. cereus* may establish an infection in mammalian tissue where it may cause severe infections, including non- gastrointestinal infections (eye, respiratory tract and nosocomial infections in hospitals) and gastrointestinal infections where it may cause food poisoning [6].

The food poisoning capabilities of *B. cereus*, reviewed by Stenfors Arnesen et al. [8], is due to synthesis of specific toxins. The emetic type of food poisoning is caused by a dodecadepsiptide, cereulide, while the diarrheal type is linked to three different toxins, haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (cytK).

*B. cereus* is regarded as one of the most common organisms that impair the quality of dairy products [18], and it is hypothesised that the highly adhesive properties of the *B. cereus* endospores are the main cause, leading to adherence of the bacterium to milk tanks and other food processing equipment, in combination with the ability of some strains to form stable biofilms at several different surfaces and under different conditions [19]. *B. cereus* carries several plasmids, but the function of these is not fully understood.

### 1.2.2 *Bacillus thuringiensis*

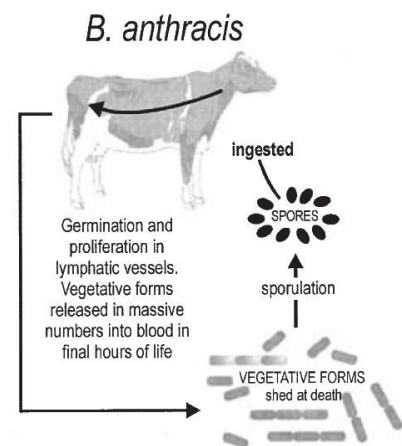
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*Bacillus thuringiensis* is a ubiquitous bacterium deposited in the soil and which produces insecticidal  $\delta$ - endotoxins during sporulation [20], hence its application as a biopesticide [21]. The toxins may contribute up to 25% of the total mass of the sporulated cells [22]. The genes encoding these toxins are located on large transferable plasmids, the loss of which makes *B. thuringiensis* indistinguishable to *B. cereus* [7]. *B. thuringiensis* carries the same chromosomal toxin genes as *B. cereus*, and may cause disease such as food poisoning [23].

Briefly, the life cycle of *B. thuringiensis*, consists of spores deposited in the soil, but introduction to the gut of earthworms, leather jacket larvae and plant rhizospheres result in germination and proliferation. Upon host death, the vegetative cells sporulate and return to the soil [5]. It has been speculated that insect larvae are the natural habitat for both *B. thuringiensis* and *B. cereus* [5].

### 1.2.3 *Bacillus anthracis*

*Bacillus anthracis* is the causative agent of anthrax disease in humans and animals, and has been identified sporadically worldwide. *B. anthracis* normally exists as a dormant spore in soil, but is capable of infecting most mammals. During the infection process, *B. anthracis* germinates, causing local infection of skin lesions (cutaneous anthrax), gastrointestinal infection or systemic anthrax through inhalation. The latter is the most severe form of disease, and is the main reason *B. anthracis* has become somewhat infamous due to its application as biochemical weapon. Upon host death, the spores are released back into the soil, completing the life cycle (figure 2) [5].



**Figure 2: Life cycle of *B. anthracis*. Figure taken from Jensen et al. [5]**

*B. anthracis* strains contain two large plasmids, pXO1 and pXO2, which contain virulence factors such as toxin and capsule genes, respectively. pXO1 encodes three different toxins; protective antigen (PA), lethal factor (LF) and oedema factor (EF), while plasmid pXO2 encodes a poly- D glutamic acid capsule which is important for pathogenicity; as it enables the bacterium to evade detection by the immune system in the infected host [5]. Loss of pXO2 results in severely decreased ability to establish an infection, and is the basis for the attenuated vaccine with the avirulent strain *B. anthracis* Sterne [5].



#### 1.2.4 *B. mycoides*

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Strains of *B. mycoides* were initially differentiated from *B. cereus* and *B. thuringiensis* by its non-motile behaviour and ability to form rhizoidal colonies. Comparison of DNA relatedness between the *B. mycoides* type strain NRRL NRS- 273 and selected strains of *B. cereus*, *B. thuringiensis*, and other strains of *B. mycoides* ranged from 24 to 34%, from 29 to 37%, and from 20 to 100% [24]. This study concluded that *B. mycoides* are not closely related to *B. cereus* and *B. thuringiensis*, therefore arguing for distinction as a separate species. Further analysis revealed that *B. mycoides* strains could be categorized into two groups, since then one of these groups has been classified as an independent species in the *B. cereus* group, *B. pseudomycoides* (1.2.5). These two groups were differentiated mainly based on their different fatty acid composition [24, 25].

#### 1.2.5 *Bacillus pseudomycoides*

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*B. pseudomycoides* was initially classified as a subgroup of *B. mycoides*, with approximately 98% identity in 16S rRNA sequences [25]. Studies of fatty acid composition [24], however, revealed differences in 12:0 iso, and 13:0 anteiso fatty acids. These results suggested a new species in the *Bacillus* group.

### 1.2.6 *Bacillus weihenstephanensis*

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A sixth species in the *B. cereus* group was proposed in 1998 [26], *Bacillus weihenstephanensis* which consist of psychrotolerant isolates. This was based on specific sequence differences in 16S rDNA and 23S rDNA, as well as in the 16S-23S rDNA spacer region and the structural genes encoding the major cold-shock protein homologue *cspA* relative to mesophilic and psychrotolerant strains previously identified as *B. cereus* or *B. mycoides*. The type strain, WSBC 10 204T, can be differentiated from *B. cereus* by growth at 7°C or lower, no growth at 43°C and specific sequences in 16S rRNA and *cspA* genes. *B. weihenstephanensis* is considered a potential pathogen [26].

### 1.2.7 *Bacillus cereus* subsp. *cytotoxis*

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The *Bacillus cereus* strain NVH391-98, later suggested as *Bacillus cereus* subsp. *cytotoxis*, was, after sequence analysis revealed several differences in gene regulation from other members of the *Bacillus cereus* group, proposed as a new species, *Bacillus cytotoxicus* [27]. The status as a novel species is yet to be approved. The strain was discovered after a serious food poisoning outbreak [28] and sequence analysis showed that the strain lacked  $\sigma^B$ , which is unique to spore-forming *Bacillus* strains, contained no tryptophan synthesis genes and contained genes implicated in biosynthesis of a food-poisoning toxin, cytotoxin K, which has been postulated to constitute the main virulence factor [27, 29]. Later it has been discovered that many *B. cereus* group strains carry the *cytK* gene, and that the severe toxicity of the NVH391-98 strain is probably due to high production of the *cytK* cytotoxin [30].

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## 1.3 Biofilm

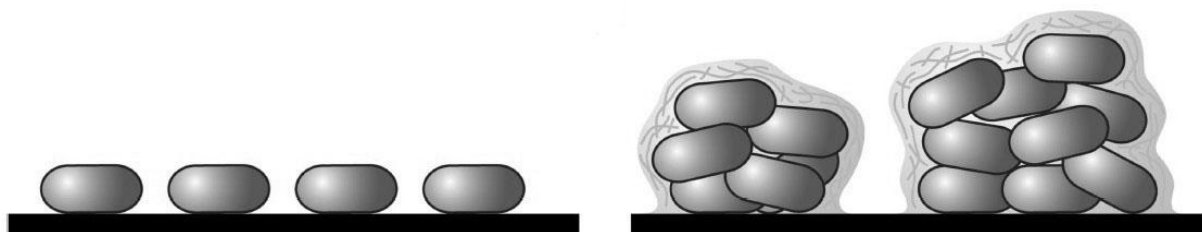
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### 1.3.1 Biofilm formation

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Biofilms are multicellular aggregates or communities of microorganisms attached to a surface [31]. Biofilms have some general properties; they usually contain an extracellular matrix consisting of an extracellular polysaccharide (EPS), nucleic acids and/or proteins. Also, biofilm formation is triggered as a response to extracellular signals from the environment and may provide protection from antibiotics, predators and components of the immune system inside a host [32].

Biofilms may be mono-, bi- or multilayered and have complex three- dimensional structures (figure 3) [32]. Monolayer formation dominates when cell-surface interaction is more prevalent than cell- cell interaction and vice versa. Biofilms can form on environmental abiotic surfaces such as minerals, on the carapaces of dead organisms, or at air-water interfaces. They can also form on biotic surfaces in the natural environment, such as plants, other microbes, and animals.



**Figure 3: Left figure shows a monolayer biofilm attached to a surface, while two clusters (multilayer) of bacteria attached to a surface can be seen on the right. Multilayer biofilms are comprised of cells and an extracellular matrix with proteins, exopolysaccharides and/or nucleic acids (figure taken from Karatan et. al [31]).**

The general model for biofilm formation is different for motile and non- motile bacteria [32]. Non- motile bacteria usually initiate their biofilm formation through individual cells starting to produce surface adhesins. This promotes cell- to- cell interaction that is important for attaching to a surface and to other bacteria [32]. Other important components for the biofilm formation, exopolysaccharides (EPS) in particular, are also produced early in the process.

Motile bacteria are able to move close to a surface and undergo several changes. The cells may switch from expressing genes important for motility (e.g. flagella) to produce components important for biofilm formation such as matrix proteins and EPS [32, 33]. Early in the process, motility has been shown to be very important for the ability of these cells to produce a biofilm.

Lemon et. al [32] states that motile bacteria develop biofilm in five distinct phases:

1. Bacteria relocate/adhere to a surface
2. Monolayer formation
3. Multilayer formation (multiple colonies in layers)
4. Extracellular matrix production
5. Biofilm stabilisation

Just as bacteria might form a biofilm, and thereby adapt to a non- motile lifestyle, they may also disperse from the biofilm and return to a motile lifestyle when this is favourable (e.g. during depletion of nutrients or accumulation of toxic products due to high cell densities) [31].

### 1.3.2 Biofilm formation in *B. cereus* and *B. subtilis*

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Several strains of *Bacillus cereus* have been shown to constitute biofilm production in low-nutrition media [18]. *B. cereus* biofilm formation has been observed on plastic, glass wool, and stainless steel, materials which have all been utilized for *in vitro* research on *B. cereus* biofilm formation [34-36]. The extracellular components making up the biofilm produced by *B. cereus* have not been heavily investigated, however, extracellular DNA (eDNA) has been shown to be a necessary component in *B. cereus* biofilms [37]. This is not unexpected since most characterized biofilms studied in other bacteria include exopolysaccharides, proteins and eDNA. eDNA has been observed in biofilms produced by other gram positive bacteria such as *Staphylococcus aureus* [38].

*Bacillus subtilis*, a model species for gram-positive bacteria, have been extensively studied for its biofilm and sporulation capabilities. *B. subtilis* tends to form pellicles of cells at the air- liquid interface in standing cultures after reaching a certain cell density [32]. Aerial projections formed after 5+ days of growth have been shown to be the preferential site of spore formation, on the basis of high expression of the gene *sspE* which has been shown to be expressed late in the sporulation process [39]. Characterization of biofilms produced by the natural wildtype *B. subtilis* strain 3610 showed that *eps* (genes responsible for exopolysaccharide production/*tasA* (gene responsible for production of the matrix protein TasA) mutants were not able to produce a proper biofilm [40]. This study underlined the importance of exopolysaccharides and TasA as matrix components.

### 1.3.3 Regulation of biofilm formation

---

Regulation of biofilm formation is highly complex and varies between different species of bacteria. A broad overview is given in a review by Karatan concerning research on biofilm formation and regulation *in vitro* [31], with an emphasis on the development of multilayer biofilms. Regulation was split into seven categories; mechanical signals, nutritional and metabolic cues, inorganic molecules, osmolarity, host- derived signals, antimicrobials and quorum signals.

Mechanical signals; there are some evidence that bacteria are able to sense and initiate lifestyle changes/biofilm formation in close proximity to surfaces. The flagellar motor system has been identified as one possible regulator of this process since defective flagella in mutant strains results in less biofilm formation *in vitro* for the motile bacterium *Vibrio cholerae* [41].

Nutritional and metabolic cues; observations and studies of biofilm formation *in vitro* have shown that the growth medium, especially the amount of nutrition, e.g. glucose levels have an effect on biofilm formation. Some species, such as *V. cholerae*, may initiate biofilm formation in environments with high concentration of glucose [42] while other species repress biofilm formation [43-45]. Catabolite repression (utilization of one carbon source at a time, preferably glucose) has also been shown to affect biofilm formation for several species.

Inorganic molecules; levels of iron and phosphate in the environment surrounding the bacteria have both been shown to affect biofilm formation for some species such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively [46, 47].

Osmolarity; increased levels of salt and/or sucrose in growth medium has been shown to inhibit biofilm formation in *P.fluorescens* [48]. Salt levels has, indirectly, proven to affect expression and activity of several biofilm-related genes [31].

Host- derived signals; defense mechanisms of the host, such as bile acids and hydrogen peroxide secretion, may promote biofilm formation in *V. cholerae* and *P. aeruginosa*, respectively [49, 50].

Antimicrobials; several studies have shown that some species alter biofilm formation when antibiotics are present, and that biofilms might explain the elevated antibiotic resistance observed by some bacteria [51].

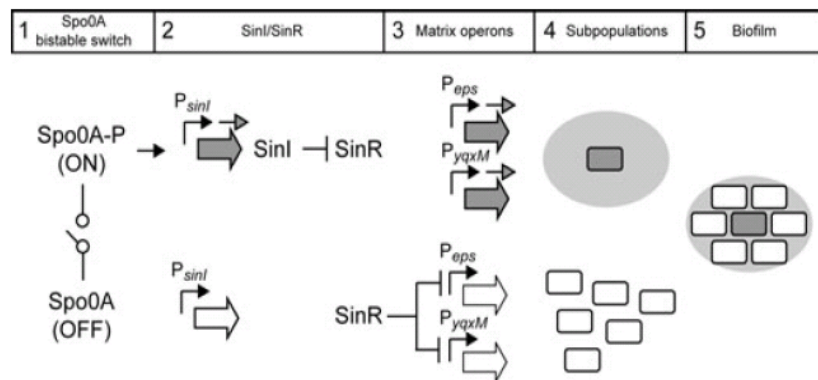
Quorum signals; quorum sensing is a signal system which enables the bacterium to change its metabolism in response to extracellular events such as high cell density or the presence of other species of bacteria. Most gram-negative bacteria have been shown to possess a system similar to the LuxI/LuxR system [52] while gram positive bacteria usually have a system where a species-specific autoinducing peptide is processed, pumped out of the cell and bind sensor kinases which phosphorylate response regulator proteins and thereby affect gene expression [52]. It has been shown that several species change biofilm formation in response to changes in cell density, such as *V. cholerae*, *S. aureus*, *P. aeruginosa* [31] and *B. cereus* [34].

#### 1.3.4 Regulation of biofilm formation in *B. cereus* and *B. subtilis*

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The regulation of biofilm in *Bacillus cereus* is not fully elucidated. More research has, however, been done on the model species *Bacillus subtilis*. Over the years, researchers have been able to identify some key genes and regulatory systems which affect biofilm formation. Studies of *B. subtilis*, which is related to *B. cereus*, show that biofilm formation predominates in low glucose environments [53], which is known to affect the positive transcription regulator Spo0A, probably due to metabolism of glucose to acetoin [54].

Spo0A is known to regulate the transcription factors SinI and SinR (often referred to as the master regulators for the switch between motile, vegetative and non-motile, sedentary behavior). SinI/SinR regulate the expression of two operons, *EpsA-O* (important for production of EPS) and *yqxM-sipW-tasA* (three genes which code for two secreted matrix proteins), which are important for biofilm formation (see figure 4) [55]. It was shown that *sinI* expression varies considerably within cells in a biofilm. This has led to a discussion whether the matrix produced in a biofilm is produced by all cells to some degree or if a subgroup of cells produce matrix components while most cells do not [56]. The possibility of such a division has been ratified by *in vitro* studies done on other bacteria such as *V. cholerae* [57]. In this study, mixtures of biofilm positive and negative cells were able to produce biofilms with 10% or less biofilm positive cells.



**Figure 4: The regulation of expression of genes in *B. subtilis* by the bistable regulator Spo0A. When Spo0A is activated, SinI, the repressor of SinR, is expressed. SinI alleviate SinR repression of several operons, *eps*, which produce extracellular polysaccharides, and *yqxM-sipW-tasA*, which produce two matrix proteins. Expression of these components have been shown to be vital for stable biofilm formation. Figure taken from Kearns et. al [56].**

Another factor in *B. subtilis*, EpsE, acts as a molecular clutch that immobilizes the flagellum by binding to a flagellar switch protein, FliG. Interestingly, the *epsE* gene is located in an operon that codes for matrix components, suggesting that interruption of motility may promote production of biofilm components [33]. Finally, sigma H ( $\sigma^H$ ) is a starvation-activated transcription factor which is required for *B. subtilis* developing complex structures in biofilms [39].



It is known that *B. cereus* strain may encode a quorum sensing system in which autoinducer-2 (AI-2) is a regulator [34]. Elevated levels of AI-2 resulted in lower biofilm production as well as dispersion of cells from a preformed biofilm.

Also, the pleiotropic regulator PlcR, which influence the expression of at least 45 genes in *Bacillus cereus* [58] by binding to an upstream palindromic motif, have been observed to influence biofilm formation *in vitro*. PlcR is regulated by a small peptide called PapR which is itself regulated by PlcR, making it a quorum- like signal system [59]. It has been shown that biosurfactants may be essential for biofilm formation in *B. cereus*, which is directly, or indirectly, regulated by PlcR [18]. A *B. cereus* ATCC14579  $\Delta$ PlcR- mutant produced >4x more biofilm compared to wt in a low- nutrient medium (EPS) ( $p < 0.05$ ) [18].

Finally, the *codY* gene is recognised to affect biofilm formation by *B. cereus*, as a *codY* mutant developed four times less biofilm compared to wt *in vitro* and detached earlier from preformed biofilms [60]. The mechanism of action for the codY protein is largely unknown. It was shown that codY repressed the production of a 130 kDa protease, but its properties have not been thoroughly investigated.

### 1.3.5 Screening biofilms in the lab

---

It is difficult to measure biofilm formation *in vivo* in humans or animal subjects. Most, if not all, biofilm research has been done *in vitro* in the lab. Today, there exist several *in vitro* methods for studying biofilms. None of these are recognized as superior to the others [32]:

Flow cells are small chambers with transparent surfaces where submerged biofilms may be formed and receive nutrition continually. The biofilms can be easily observed with confocal scanning laser microscopy [61]. The strength of this particular approach is the possibility to

observe biofilm formation over time. The weakness when using flow cells is that high-throughput screening of different strains can be time consuming and difficult

Floating pellicles that form in the liquid- air- interface in standing cultures may also be used for screening of different strains, and it is also an option to grow colonies on agar plates. Colonies which exhibit macroscopic, complex architecture are recognised as biofilms and may be easily detected by visual inspection [61] . This method may also be used for high-throughput screening.

Finally, microtiter plate based screening techniques can be useful to study submerged or air-liquid interface biofilms in a no- flow system. Microtiter plate assays are useful for high-throughput screening, and a number of genes involved in biofilm formation have been discovered by this method [32, 62]. This method was chosen for work in this thesis since it is relatively fast to perform on a range of different strains at the same time.

## **1.4 Genomics relevant to the thesis**

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### **1.4.1 Riboswitches**

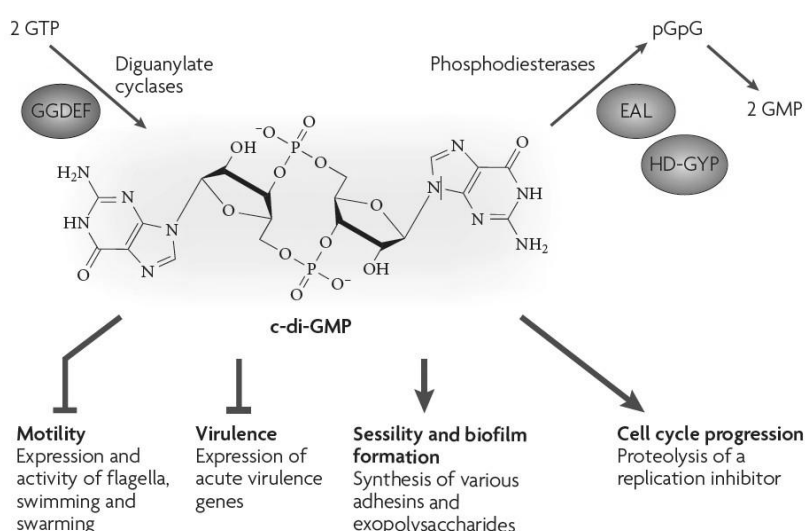
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Riboswitches are typically RNA domains in the 5- untranslated region of certain mRNAs adjacent to open reading frames (ORF) that may alter gene expression in response to changing concentrations of a specific ligand [63]. The riboswitch domain forms a three-dimensional structure with receptor functions which has been shown to bind ligand with high affinity and specificity [64]. A whole range of riboswitches have been identified in different bacteria, including riboswitches that sense guanine, lysine, adenine, vitamin B12 and other ligands. One such domain is GEMM [65] which has been shown to possess high affinity for the second messenger c-di-GMP.

### 1.4.2 C- di- GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a second messenger found in many bacteria [66]. C-di-GMP is produced from two GTP molecules by diguanylate cyclases that carry GGDEF domains, and is degraded by phosphodiesterases (PDEs) with one of two different domains, EAL or HD- GYP (see figure 5). Most of these enzymes contain sensory input domains in the N- terminal transmembrane helices which can sense extracellular signals such as oxygen levels and light radiation, and affect enzyme activity.

Intracellular concentrations of c-di-GMP are controlled by the amount and activity of these two groups of proteins. The intracellular concentration affects the extent of binding to specific effectors, which can be either proteins or riboswitches containing a GEMM domain in the 5'- untranslated region. Targets can be promoters in DNA (if the effector is a transcription factor), enzymes or complex cellular structures, such as the flagellar basal body or exopolysaccharide synthesis [66].



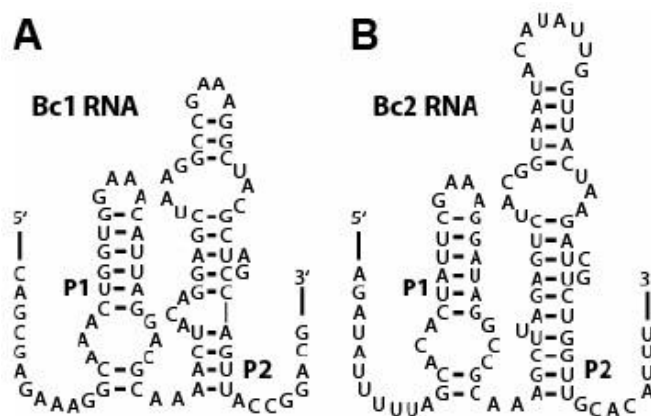
**Figure 5: Intracellular c-di-GMP level-based regulation of bacterial cellular processes, two GTP molecules produce c-di-GMP by diguanylate cyclase activity (GGDEF domain), and is degraded by phosphodiesterase activity (with EAL and HD- GYP domains) to produce pGpG, and later two GMP molecules.**

C-di-GMP is implicated in cell differentiation, conversion between motile and biofilm lifestyles (stimulation of biosynthesis of adhesins and exopolysaccharide matrix substances) and virulence gene expression in gram-negative bacteria. The role of c-di-GMP in gram-positive bacteria has, however, not been heavily investigated, and only seven proteins with GGDEF and EAL domains have been found [67]. This has led some researchers to be doubtful of c-di-GMP as a major regulator of lifestyle behaviour in gram-positive bacteria.

## Chapter 2: Background for the thesis

In 2008, Sudarsan et al [63], identified a highly conserved RNA domain, GEMM. GEMM resides upstream of genes encoding DGC and PDE proteins in various organisms and upstream of genes that are known to sense the intracellular signal molecule cyclic di- GMP (1.4.2). The high conservation and genomic distributions of GEMM RNAs are characteristic of riboswitches (1.4.1), RNA domains in mRNA that may control translation.

In their paper, the researchers prepared transcriptional fusions for c-di-GMP riboswitches from *Bacillus cereus* called Bc1 and Bc2 (figure 6), and cloned the constructs into a *Bacillus subtilis* host strain. The expression patterns, using a  $\beta$ -galactosidase assay, revealed that Bc1 wildtype showed high activity while the isogenic mutant M3 did not. The Bc2 wildtype showed low activity while the isogenic mutant M3 showed high activity. These results indicated that Bc1 acted as an inducer of downstream gene expression (c-di-GMP-responsive “on” switch) whereas Bc2 acted as a repressor of downstream gene expression (c-di-GMP- responsive “off” switch) [63].



**Figure 6: Bc1 and Bc2 RNA sequence and 2D structure. Figure taken from Sudarsan et al. support material [63]**

It has been shown that c-di-GMP is implicated in the change from planktonic to a sedentary lifestyle, in biofilm-forming phase in bacteria such as *Vibrio cholerae* [68]. Putative role of c-di-GMP in regulation of biofilm formation in gram-positive bacteria is, however, not elucidated. Following the publication of the paper, DNA and protein searches were conducted by BLAST [69] to investigate whether these two riboswitches were present in a range of different strains in the *Bacillus cereus* group (Annette Fagerlund, personal communication). In one strain, known to be a stable biofilm producer, *Bacillus thuringiensis* 407, both riboswitches were present in the genome sequence.

*B. thuringiensis* 407 Cry<sup>-</sup> (Bt 407) was chosen as model organism for studying biofilm formation due to its well-known ability to produce biofilms and earlier experience in the lab. It is a model strain used in many previous studies of *B. thuringiensis* genetics and has recently been sequenced (taxid: 527021). The Bt 407 wildtype was isolated in Brazil by Dr. Sergio Batista Alves from an insect larva (*Anagasta Kuhnella*). It was later cured of plasmid encoding insecticidal crystal toxin by heat treatment by Olivia Arantes (Cry<sup>-</sup>). Both wild type and mutant strains were first described in 1989 [70]. The parental strain was serotyped at Institut Pasteur, and was defined as belonging to serotype H1 (*Bacillus thuringiensis* serovar *thuringiensis*). However, the serotyping of the 407 Cry<sup>-</sup> strain did not allow distinguishing this strain from a *B. cereus* strain (unpublished data). Plasmid analysis revealed the presence of at least six plasmids (ranging from about 4 to 70 kb) in the wildtype strain, Bt 407 Cry<sup>+</sup>. This analysis also showed that strain Bt 407 Cry<sup>-</sup> had lost the largest plasmid of about 70 kb (Olivia Arantes and Didier Lereclus, unpublished data).

The Bc1 riboswitch sequence was identified to be upstream from a gene, bhthur0002\_3430 (locus tag), which will be referred to as Bt407\_0422 in this thesis, and which is an ortholog of BC0422 in *B. cereus* ATCC 14579. This open reading frame is annotated to contain methyl- accepting chemotaxis protein properties. The Bc2 riboswitch was similarly identified at the end of a contig with no adjacent ORF in *B. thuringiensis* 407. However, an ORF was identified by manual inspection, and identified at the end of the contig and continuing on another contig. PCR, using genomic DNA from *B. thuringiensis* 407 as template, and subsequent sequence analysis of the PCR product closed the gap between the two contigs, revealing the sequence of the gene. The gene was identified to be an ortholog to BC1060 in

*B. cereus* ATCC 14579. BC1060 is annotated to contain collagen- binding adhesion protein properties. This gene will be referred to as Bt407\_1060 in this thesis.

In order to control the Bt407\_0422 and Bt407\_1060 proteins for their effect on biofilm formation, Annette Fagerlund prepared gene deletion mutant *B. thuringiensis* 407 strains of both genes and did a preliminary biofilm assay which showed that biofilm formation was significantly less in the Bt407\_1060 deletion mutant and possibly decreased during the first 24 hours in the Bt407\_0422 deletion mutant compared to the *B. thuringiensis* 407 wildtype strain (unpublished results). The aim of this thesis was to expand on the initial investigation. The initial objectives were:

- Expand biofilm formation screening of the Bt407\_1060 and Bt407\_0422 gene deletion mutants.
- Clone promotor regions of Bt407\_0422 and Bt407\_1060 in pHT304-18z (lacZ reporter vector) in order to do lacZ assays.
- Clone the two genes, Bt407\_0422 and Bt407\_1060, into the pHT304-pXyl expression vector for overexpression and complementation assays for the gene deletion mutants.

When the constructs were complete, the aim was to perform different assays and collect data relevant to the role of these two genes in biofilm formation. The initial aims would be expanded on if time allowed it.

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## Chapter 3: Materials

### 3.1 Bacterial strains

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Strains	Note(s)
<i>Bacillus thuringiensis</i> strain 407 (Bt 407)	Further information in chapter 2
<i>Escherichia coli</i> ( <i>E. coli</i> ) XL-1 Blue	Manufacturer: Stratagene.
<i>Escherichia coli</i> ( <i>E. coli</i> ) electrocompetent cells (TOP10)	Manufacturer: Invitrogen

**Table 3.1: Overview of bacterial strains used in the thesis**

### 3.2 Vectors (plasmids)

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Vectors	Characteristics	Reference
pHT304-18z	pHT304 containing <i>lacZ</i> promoterless gene	Arantes (1991) [71]
pHT304-pXyl	pHT304 containing <i>xylA</i> promoter	Arantes (1991) [71]
pMAD	Vector for cloning in gram-positive bacteria, contain $\beta$ -galactosidase-encoding gene and <i>erm</i>	Arnaud (2004) [72]
pUC19	Shuttle vector, contains <i>lacZ</i> gene and <i>amp</i>	Yanisch-Perron et al. (1985) [73]

**Table 3.2: Overview of vectors (plasmids) used in the thesis**



### 3.3 Oligonucleotides (primers)

Primer name	Sequence (5'-3')
02561_F2	aaaggtgggaagccaaaaat
Bt407_0422-F-XbaI	ttatctagattaaggaaggtggatattaatgaaaa
Bt407_0422-R-KpnI	atcggtaccggagatatccctttgat
00330-Bt407_0422-R2	gtcaccacgaacctattca
S2_pUC19R	taacaatttcacacaggaaacagc
S1_pUC19F	gtttccagtcacgacgtt
pHT304-pXyl_F2	ggtttgatcagcgatatccac
pHT304-pXyl_forward	tccactcatccactccatttg
PU	cgccagggtttccagtcacgac
OVG	cgtaatcttacgtcagtaattccacagta
P-Bt407_0422-F	aacaagcttcgaaattcattagttaagat
P-Bt407_0422-R	ttaggatccaccttccttaattaacataaac
P-Bt407_1060-F	agactgcagcccgagttaagagaaaaag
P-Bt407_1060-R	tacggatcctttgcatagtccctcct
Bt407_0422_mut1_F	cttgaaaaaatctccaaatcagcgcaatctgttacagatcaag
Bt407_0422_mut1_R	cttgatctgtaacagattgcgctgatttggagatttttcaaag
Bt407_0422_mut2_F	caacagcagtagtaatgttgaagaaagcatgaagagcaag
Bt407_0422_mut2_R	cttgctctttcatgctttcttcaacattactactgctgttg

**Table 3.3: Overview of oligonucleotides used in the thesis**

## 3.4 Enzymes

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### 3.4.1 Restriction enzymes

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Restriction enzyme name	Manufacturer
<i>Bam</i> HI	Biolabs
<i>Hind</i> III	Biolabs
<i>Kpn</i> I	Biolabs
<i>Pst</i> I	Biolabs
<i>EcoRV</i>	Biolabs
<i>EcoRI</i>	Biolabs

**Table 1.4: Overview of restriction enzymes used in the thesis**

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### 3.4.2 Other enzymes

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Enzyme name	Manufacturer
Dynazyme II DNA polymerase	Finnzymes
pfu Ultra polymerase	Stratagene
T4 DNA ligase	Invitrogen
<i>DpnI</i>	Stratagene

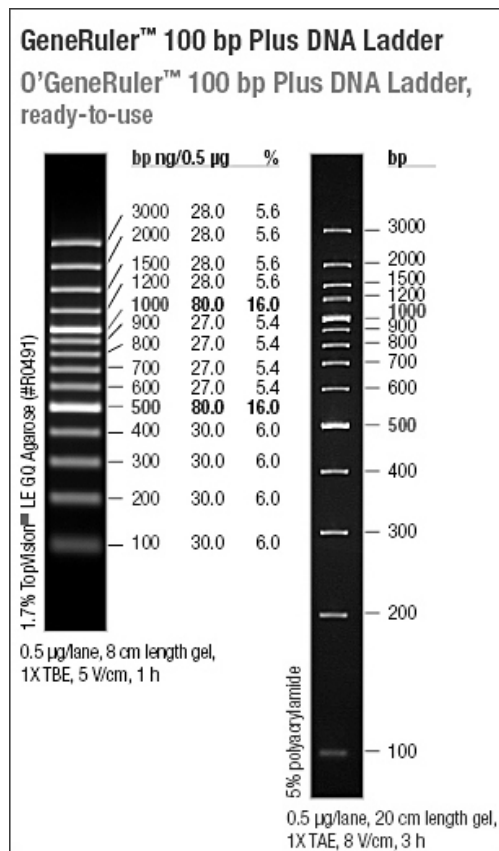
**Table 3.5: Overview of enzymes (- restriction enzymes) used in the thesis**

## 3.5 Molecular weight standards

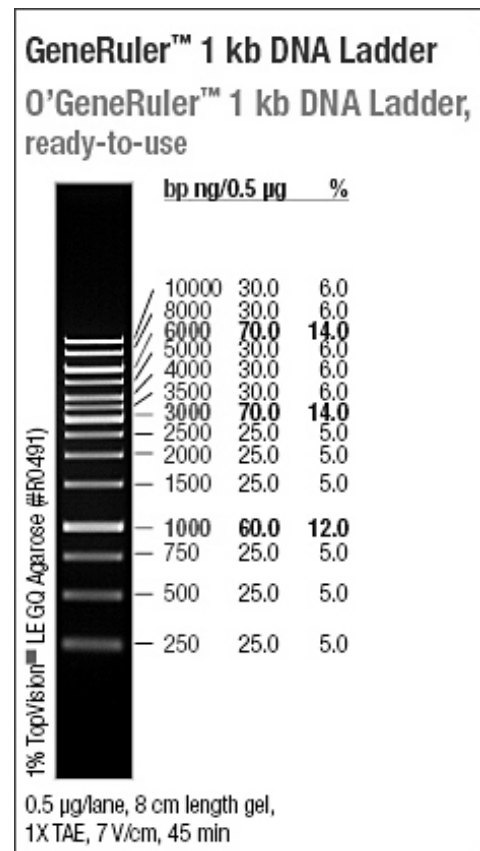
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Name	Manufacturer
100bp GeneRuler + DNA ladder (figure 7)	Fermentas
1kb GeneRuler DNA ladder (figure 8)	Fermentas

**Table 3.6: Overview of molecular weight standards used in the thesis**



**Figure 7: GeneRuler 100bp  
 Plus DNA ladder**



**Figure 8: Generuler 1kb DNA  
 ladder**

## 3.6 Chemicals

Nr.1 bacterial agar (Oxoid)

Acetic acid (Merck)

Acetone (J.T Baker)

Agarose (Sigma)

Ampicillin (Sigma)

Bactopeptone (Oxoid)

Bromophenol blue (Sigma)

Boric acid ( $\text{H}_3\text{BO}_3$ ) (Sigma)

Crystal violet (Sigma)

Di- Sodium hydrogen phosphate 2- hydrate ( $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ ) (Merck)

dNTP (Qiagen)

Erythromycin (Sigma)

Ethidium bromide (EtBr) (Sigma)

Ethanol ( $\text{EtOH}$ ) (Arcus)

Ethylenediaminetetraacetic acid (EDTA) (M&B)

Hydrochloric acid ( $\text{HCl}$ ) (Prolab)

Isopropanol (Arcus)

Kanamycin (Arcus)

Methanol (Prolab)

Magnesium chloride ( $\text{MgCl}_2$ ) (Merck)

Magnesium sulphate ( $\text{MgSO}_4$ ) (Merck)

Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) (Merck)

Potassium chloride ( $\text{KCl}$ ) (Merck)

Sodium chloride ( $\text{NaCl}$ ) (JT Baker)

Tetracyclin (Arcus)

Tris base (Sigma)

Tryptone (Oxoid)

Yeast extract (Oxoid)

**Note: Chemicals provided in the commercial kits are not included in this list.**

### 3.7 Commercial kits

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E.Z.N.A.<sup>®</sup> Gel Purification Kit (Omega Bio-Tek)

E.Z.N.A.<sup>®</sup> Plasmid Miniprep Kit I (Omega Bio-Tek)

QIAprep Spin Miniprep Kit (50) (Qiagen)

QIAGEN Plasmid Midi Kit (25) (Qiagen)

QIAquick PCR Purification Kit (50) (Qiagen)

QIAquick Gel Extraction Kit (50) (Qiagen)

### 3.8 Miscellaneous

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0.25µm filter paper (Millipore)

Eppendorf tubes (2ml) (Treff lab)

NUNC tubes (15 and 50ml) (Corning)

8x PCR tubes (0,2ml) with lids (Axygen)

Round- bottomed PVC microtiter plates with 96 wells (Falcon 353911)

Sterilin tubes (15ml) (Sterilin)

0.2cm electroporation cuvettes (Bio-Rad)

## 3.9 Growth media

Lysogeny Broth medium (LB)

---

10g tryptone

5g yeast extract

10g NaCl

Dissolved in ~900ml dH<sub>2</sub>O, pH adjusted to 7.0 with HCl and total volume adjusted to 1000ml with dH<sub>2</sub>O. Autoclaved and stored at 4°C.

LB agar plates

---

10g tryptone

5g yeast extract

10g NaCl

15g NR 1 bacterial agar

Dissolved in ~900ml dH<sub>2</sub>O, pH adjusted to 7.0 with HCl and volume adjusted to 1000ml with dH<sub>2</sub>O. Autoclaved and stored at 4°C.

### Bactopeptone medium

---

10g bactopeptone

5g yeast extract

10g NaCl

Dissolved in ~900ml dH<sub>2</sub>O, pH adjusted to 7.0 with HCl and volume adjusted to 1000ml with dH<sub>2</sub>O. Autoclaved and stored at 4°C.

### Super Optimal broth with Catabolite repression (SOC) medium

---

20g tryptone

5g yeast extract

0.5g NaCl

0.18g KCl

0.95g MgCl<sub>2</sub>

1.2g MgSO<sub>4</sub>

3.6g glucose

Dissolved in 1000ml dH<sub>2</sub>O and pH adjusted to 7.0 with NaOH. Autoclaved, aliquoted and stored at -20°C.



## 3.10 Buffers and solutions

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0.3% crystal violet solution (for biofilm assay)

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0.6g crystal violet

Dissolved in 200ml dH<sub>2</sub>O over night, then sterile filtered and stored at room temperature.

6x Loading buffer (for agarose gel electrophoresis)

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15ml glycerol

0,125ml bromophenol blue

Volume adjusted with ~20ml TBE buffer and dH<sub>2</sub>O to 50ml total volume. Prepared by Annette Fagerlund, aliquoted for own use.

#### Aceton/ethanol (1:4) (for biofilm assay)

---

250ml acetone

750ml ethanol

Mixed and stored at room temperature in sealed container

#### 50x TAE buffer (Tris/Acetate) (for gel electrophoresis)

---

242 g tris base

57.1 ml acetic acid

100ml 0.5M EDTA (pH 8.0)

Dissolved in 1000ml dH<sub>2</sub>O and stored at room temperature.

#### PBS (for biofilm assay)

---

16g NaCl

0.4g KCl

0.54g Na<sub>2</sub>HPO<sub>4</sub> x 2H<sub>2</sub>O

3.56g KH<sub>2</sub>PO<sub>4</sub>

Dissolved in 1900ml dH<sub>2</sub>O, pH adjusted to 7.4 with HCl. Total volume adjusted to 2000ml with dH<sub>2</sub>O. Autoclaved, stored at room temperature in a suitable bottle.

#### 10x TBE buffer (Tris/Borate) (for gel electrophoresis)

---

108g tris base

55g boric acid

9.3g EDTA

Dissolved in 950ml dH<sub>2</sub>O. Final volume adjusted to 1L with dH<sub>2</sub>O in an erlenmeyer bottle.  
Stored at room temperature for further dilution into working concentration (1x).

#### 50x TAE buffer (Tris/Acetate) (for gel electrophoresis)

---

242 g tris base

57.1 ml acetic acid

100ml 0.5M EDTA (pH 8.0)

Dissolved in 1000ml dH<sub>2</sub>O and stored at room temperature.

## Chapter 4: Methods

### 4.1 Growth of bacteria

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#### 4.1.1 *Escherichia coli*

---

*Escherichia coli* (*E. coli*) was an essential bacterium in the cloning procedure. *E. coli* was grown in either LB medium or on LB agar plates at 37°C, the optimal growth temperature for this bacterium. Liquid cultures were subject to rotation at ~220 rpm throughout incubation.

#### 4.1.2 *Bacillus cereus*

---

The different strains of *Bacillus cereus* used in this thesis were grown either in LB or on LB agar plates at 30°C, the optimal growth temperature for this bacterium ranges between 28 and 35°C [17] . Liquid cultures were subject to a rotation of ~220 rpm throughout incubation.

## 4.2 Preparation of cells for electrotransformation

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### 4.2.1 Electrocompetent *E. coli*

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Electrocompetent *E. coli* was made for use in the cloning procedure due to its well-known properties regarding transformation of plasmids and fast growth rate.

#### Protocol

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An appropriate amount of glycerol stock *E. coli* XL-1 Blue were streaked onto a 20µg/ml tetracyclin LB agar plate and incubated at 37°C over night. One colony was picked and used to inoculate 50 ml LB medium which was grown at 37°C (220rpm) over night. 10ml of the inoculate was transferred to two erlenmeyer bottles, each containing 1000ml LB medium and incubated at 37°C (220rpm) until an OD600 value of 0.4- 0.5. Cells were transferred to four 500ml centrifuge tubes and chilled on ice for 20 minutes.

The cultures were then centrifuged for 3750x g for 10 minutes at 4°C in a Beckman JA-10 rotor. The supernatant was then removed by turning the tubes upside down and draining on filter paper for a short time. The pellets were then resuspended in 10ml ice-cold MilliQ dH<sub>2</sub>O, and transferred to two centrifuge tubes. After adding 340ml ice-cold MilliQ dH<sub>2</sub>O, the tubes were gently mixed and centrifuged as before. This step was repeated three times.

After washing, the pellets were resuspended in 10ml ice- cold 7% DMSO. The suspensions were then transferred to a 50ml NUNC- tube and the original centrifuge tubes were washed thoroughly with another 10ml 7% DMSO and transferred to the NUNC tube (total volume 40ml). The NUNC tubes with cell suspensions were then centrifuged at 3750x g for 10

minutes in a Sorvall RC3C centrifuge. The pellet was resuspended in 4ml 7% DMSO, the suspension was dispensed into pre- chilled eppendorf tubes (80µl aliquot) and then frozen on a dry ice/ethanol bath prior to transfer and storage at -80°C. The cells were tested for transformation effectiveness (5.3).

#### 4.2.2 Electrocompetent *Bacillus cereus*

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Electrocompetent *Bacillus cereus* was made in small batches for immediate transformation of different reporter vectors for use in the biofilm assay.

##### Protocol

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The strains to be used for transformation was plated out on LB plates (with appropriate antibiotics) and incubated at 30°C overnight. One colony was then transferred to 5ml LB medium (with appropriate antibiotics) and incubated at 30°C and ~220rpm overnight.

The overnight culture was diluted 1:200 in 200ml LB medium in a 1L erlenmeyer bottle and incubated at 30°C until an OD600 of 0.4- 0.6. The cultures were harvested by centrifugation at 4°C, 4000x g for 10 minutes in a Sorvall RC3C centrifuge. The pellets were washed three times with 2ml and once with 1ml 10% glycerol and spun down for 1 minute at 8000rpm (4°C) between each wash in a Thermo Heraeus BioFuge table top centrifuge. The cells were finally resuspended in an appropriate volume of 10% ice cold glycerol. The cells were used for transformation the same day.

## 4.3 Preparation of purified plasmid DNA

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Purification of DNA from cell suspensions and liquid reactions was done extensively for this thesis. Commercial kits (3.7) from Qiagen and Omega Bio-Tek (E.Z.N.A) were used for this purpose, and protocols issued from the manufacturer were followed. The kits are all based on the same principle for purification of DNA. If the DNA content was located inside bacteria, an alkaline lysis based on the method of Birnboim and Doly [74] was applied. The lysate was then neutralized and adjusted to high- salt binding conditions in one or two steps prior to application onto a silica membrane for DNA absorption.

However, if the DNA content was located in a liquid solution (e.g. PCR reactions), the volume was adjusted to 100µl and applied directly onto a silica membrane for DNA absorption. After 2-3 washes with different buffers to remove RNA, cellular proteins, and other metabolites, a low- salt buffer with appropriate pH (usually between 7.0 and 8.5) was used to elute the purified DNA from the silica membrane into an eppendorf tube for use in other procedures.

### 4.3.1 PCR purification

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PCR purification was done with EZNA Gel Extraction kit and Qiagen PCR purification kit (3.7). Protocols provided by the manufacturer were followed [75, 76].

### 4.3.2 Miniprep

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Minipreps of plasmid DNA were prepared with EZNA Miniprep kit and Qiagen miniprep kit (3.7). Protocols provided by the manufacturers were followed [77, 78].

### 4.3.3 Midiprep

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Midipreps of plasmid DNA was prepared with Qiagen Midiprep kit (3.7). Protocol provided by the manufacturer was followed [77].

## 4.4 Determination of DNA concentration in free solutions

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Several stages in the cloning procedure depended on knowledge of the DNA concentration in liquid solutions. Two different techniques were used for determining DNA concentrations, spectrophotometry and/or quantitative electrophoresis.

### 4.4.1 Spectrophotometry

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Absorption spectroscopy of nucleic acids is carried out at 260nm (absorption spectra maximum), 1 unit corresponds to ~50µg/ml DNA. To estimate the purity of the solution (especially amount of protein contamination), the sample is usually also measured at 280nm



(absorption spectra maximum for aromatic amino acids) and a 260/280 ratio is calculated. A 260/280 ratio of 1.8 indicates 100% DNA and 0% protein [79]. Spectrophotometry was carried out using either Eppendorf Bio Photometer or PicoDrop using Picodrop v2.07 software.

#### 4.4.2 Quantitative electrophoresis

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Spectrophotometry was used almost exclusively for samples where the concentration of DNA was expected to be high ( $>500\mu\text{g/ml}$ ). Quantitative electrophoresis was carried out when yield of DNA was expected to be uncertain/very low. This was done running several dilutions of the test sample on a gel and compare the intensity of the bands with a size marker (3.5) where the concentration was known (eg. 5 $\mu\text{l}$  100bp GeneRuler DNA ladder (Fermentas)).

### 4.5 Polymerase chain reaction (PCR)

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Polymerase chain reaction (PCR) is a well- known and common technique to produce large quantities of a specific DNA fragment in vitro [80]. PCR requires detailed knowledge of the sequence of the DNA segment for precise design of forward and reverse primers. All primers used had optimal melting point of  $\sim 55^{\circ}\text{C}$ , and length varied between 15-25 nucleotides, except primers used for mutagenesis (see 4.9).

## Protocol

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For practical application, the PCR reaction requires template DNA, forward and reverse primers, a heat- stable enzyme with DNA polymerase properties, DNA polymerase buffer, a solution containing the four different nucleotides (cytosine, thymine, adenine and guanine) and a programmable, automated heat- block.

### General setup of PCR reactions (total volume 50µl):

~50ng chromosomal DNA (usually 1-2µl of solution, either purified plasmid prep or resuspended bacterial culture for colony screening)

1µl DNA polymerase (last component added to the reaction)

1µl forward primer (20µM)

1µl reverse primer (20µM)

5µl 10x DNA polymerase buffer (appropriate for the chosen polymerase)

dH<sub>2</sub>O to 50µl

A general PCR cycle could be like this:

1. 94°C for 3 minutes, 1 cycle
2. 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, 30 cycles
3. 72°C for 3 minutes, 1 cycle, then hold at 4°C until use

It should be noted that the different temperatures and time intervals (especially for the last part of the second cycle rotation (where the polymerase copy the template)) could vary, depending on the length of the DNA segment to be replicated. PCR in the lab was done either on a Thermo PXE 0.2 Thermal Cycler or Applied Biosystems GeneAmp PCR system 2700.

## 4.6 Gel electrophoresis

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Agarose gel electrophoresis was used for separation and purification of specific DNA fragments, and was also used for quantitative analysis of DNA concentration in liquid solutions (4.4.2).

DNA fragments of different lengths are separated by several mechanisms [80] p. 16. DNA is uniformly, negatively charged, which means that it will travel towards the positive electrode in an electric field. However, DNA fragments of different sizes are not separated by their size in a free solution with electrodes at either side. A matrix where the DNA has to travel through it is important; hence the use of agarose gels. The three-dimensional composition of the agarose gel is important, especially the size of the pores. Small, flexible DNA fragments are able to migrate faster through a gel compared to longer, less flexible fragments. A gel with a high amount of agarosis is usually ideal for separating smaller DNA fragments, and vice versa.

To visualize DNA, ethidium bromide is added to the agarose gels. Ethidium bromide intercalates with the DNA, and fluoresces when subjected to UV illumination.

### Protocol

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For practical application, the agarose gels used were between 0.5 to 2%, the agarosis was added to the buffer used in the electrophoresis chamber in an erlenmeyer bottle (usually TBE, but also TAE) and heated until fully dissolved in a microwave oven. Ethidium bromide was added to the erlenmeyer bottle in a concentration of 0.5µg/ml and then poured into a gel mould for cooling.

The gel was then placed into an electrophoresis chamber, a 6x loading buffer (3.10) were added to the mixtures of DNA fragments to be separated. A suitable volume of the mixture was applied to a separate well in the gel, then ran on 60-120V (adjusted according to the size of the gel) for an appropriate time. The DNA fragments were then visualised with an UV illuminator (Biorad Gel Doc 1000) and photographed using Multi- Analyst software.

To determine size (and to some extent amount of DNA) of the different DNA fragments, different size markers were applied on the gels (3.5).

## 4.7 Enzymatical restriction digestion

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Enzymatical restriction digestion was used both for practical purposes during the cloning procedure (cutting and joining of DNA fragments) and for analytical purposes (restriction testing). A range of different enzymes were used (3.4.1), but the general setup of each reaction was essentially the same.

### Protocol

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#### General setup of restriction digest reaction (total volume 10µl):

1-2µl template (dependent on concentration of DNA)

0.5µl restriction enzyme

1µl 10x reaction buffer (specific to the restriction enzyme)

*If the particular enzyme reaction was dependent on bovine serum albumin (BSA):*

1µl 10x BSA

dH<sub>2</sub>O until 10µl

It should be noted that the amount of enzyme was changed when necessary, and this general setup did not apply for the big digests of plasmid DNA used in parts of the cloning procedure.

## 4.8 Ligation of DNA fragments

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Ligation of two DNA fragments (typically a small insert with a larger reporter vector) was done to join digested DNA fragments after DNA purification. The setup of these reactions varied with the concentration of insert and vector, but a general rule-of-thumb reaction was based on a 1:3 molar ratio between vector and insert. However, it was important to ensure that the reaction contained sufficient insert DNA, therefore, the molar ratio could vary between 1:3- 1:10.

### Protocol

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#### Ligation reaction (total volume 15µl):

1µl T4 DNA ligase (last component added to the reaction)

1.5µl 10x T4 DNA ligase reaction buffer (thawed and vortexed to ensure no precipitation)

12.5µl vector and insert mixture (depended on the DNA concentrations)

The reactions were then put in a MJ Research Programmable Thermal Controller at 16°C overnight. Next day, the reactions were dialyzed for 30 minutes on filter paper to remove salts prior to electrotransformation.

## 4.9 Mutagenesis

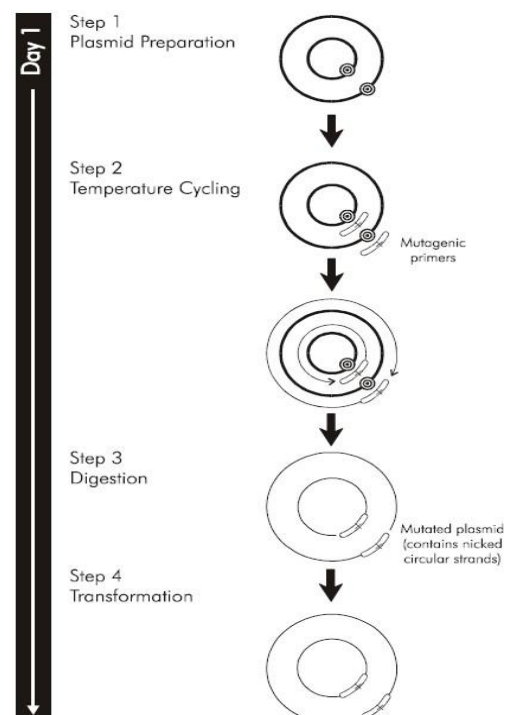
Mutagenesis can be applied when removal of mutations that could result in erroneous translation in a DNA sequence is necessary.

### 4.9.1 Primer design

Design was done after guidelines from Quikchange [81] by Annette Fagerlund. The most important features of the primers was length (between 25 and 45bp), estimated melting temperature at 78°C, a minimum of 40%GC content and termination in one or more G or C-molecules. The mutated base-pair was located in the middle of the primer with >10–15 bases of correct sequence on both sides.

### 4.9.2 Protocol

The protocol from Quikchange [81] was followed. The procedure is based on a polymerase chain reaction where the primers with the correct sequence are designed to overlap the mutation and copy the plasmid. After initial PCR reaction, both original, bacterial plasmids and newly synthesized plasmids are present.



**Figure 9: Overview of mutagenesis using PCR**

A restriction enzyme, *DpnI*, that digest target sequence 5'-Gm6ATC-3' in methylated DNA, will then digest the original plasmids while leaving the newly synthesized plasmids intact. The reaction is transformed into a proper bacterium, e.g. electrocompetent *E. coli* and sequencing of minipreps from colonies will ratify whether the mutagenesis was successful. This procedure can be done several times if more than one mutation is present.

## 4.10 DNA sequencing

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Sequencing of different constructs was outsourced to the ABI-lab at CEES, Department of Biology and Molecular Biosciences, University of Oslo, Oslo, Norway. The lab employs 3730 high-throughput capillary electrophoresis machines (Applied Biosystems).

The lab could analyse reactions with 8µl of PCR product with 2µl of a 5µM primer added, the general setup was 1-2µl miniprep (>200µg DNA) with 1µl 10mM primer and dH<sub>2</sub>O to 10µl for each reaction.

Analysis of the sequence chromatography diagrams was carried out using Sequence Scanner v1.0 (Applied Biosystems).

## 4.11 Transformation of circular DNA (plasmids) into bacteria

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Zimmerman & Vienken (1983) observed that cells can take up exogenous DNA from the surrounding environment when treated with high- voltage electric pulses [80]. It has been shown that electroporation is as, or more, effective compared to chemical transformation.

#### 4.11.1 Transformation into *E. coli*

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Transformation of plasmids into *E. coli* was done extensively in the cloning procedure. The general reaction was 1µl (if TOPO- plasmid) to 15µl (ligation reaction (4.8)) added to ~40µl electrocompetent cells.

The mixture was added to a chilled 0.2cm electroporation cuvette, dried thoroughly and it was made sure that no air bubbles were present in the mixture. The cuvette was then placed in a Biorad Gene Pulser II machine and electroporated using 2,5kV, 25µF and 400Ω. The RC time constant, an estimate of pulse length [82], was expected to be ~10msec. Immediately after applying electric shock to the cells, 500µl SOC medium was added and the mixture transferred to a 15ml Sterilin tube. Another 500µl SOC medium was added to wash out the remaining cells and added to the Sterilin tube. The cells were then incubated at 37°C (220rpm) for 1 hour before they were plated out on LB plates with appropriate antibiotics.

#### 4.11.2 Transformation into *Bacillus cereus*

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The electrocompetent cells were prepared the same day as transformation (4.2.2). The general reaction setup consisted of 50µl cells and 4µl purified plasmid (~1000-2000µg DNA). One or more reactions were prepared (depended on the effectiveness of transformation).

The mixture was added to a chilled 0.2cm electroporation cuvette, dried thoroughly and it was made sure that no air bubbles were present in the mixture. The cuvette was then placed in a Biorad Gene Pulser II machine and electroporated using 2kV, 25µF and 200Ω. The RC

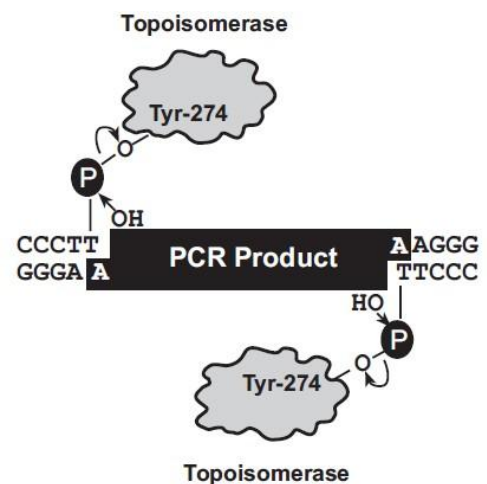


time constant, an estimate of pulse length [82], was expected to be ~4-5msec. Immediately after shocking the cells, 500µl SOC medium was added and the mixture transferred to a 15ml Sterilin tube. Another 500µl SOC medium was added to wash out the remaining cells and added to the Sterilin tube. The cells were then incubated on 30°C and ~220rpm for 2-3 hours before they were plated out on LB plates with appropriate antibiotics.

## 4.12 TOPO cloning

Insertion of a PCR product into the TOPO II plasmid was done to ease the cloning process due to the possibility of indentifying one clone with correct sequence and restriction sites.

TOPO vectors contain replication sites for *E. coli* ampicillin/kanamycin resistance genes and two distinct properties for insertion of PCR products. Single 3'-thymidine (T) overhangs for TA cloning and Topoisomerase I covalently bound to the vector (see figure 10).



**Figure 10: TOPO vector, two topoisomerases located close to the TA overhangs.**

### The TOPO cloning reaction setup:

0.5-4µl fresh PCR product  
1µl TOPO vector  
dH<sub>2</sub>O until 5µl

After mixing, the reaction was left for incubation in room temperature for 5 minutes. The entire reaction was then added to electrocompetent *E. coli* for transformation.

## 4.13 Biofilm screening assay

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Biofilm formation by *Bacillus thuringiensis* 407 was studied with a microtiter plate assay used in several studies of biofilm formation by *Bacillus cereus* and other bacteria [34, 62]. In order to screen bacterial strains with this assay, the bacteria have to form biofilm at the liquid- air interface and it needs to adhere to a plastic surface. Both these properties have to be present. The *B. thuringiensis* 407 strain used in this thesis are known to form biofilms in a low nutrition medium (e.g. bactopectone), and it readily form biofilms which adhere to plastic.

Stock strains were plated onto LB agar plates with appropriate antibiotic(s) and grown at 30°C over night. One colony were picked and transferred to 5ml LB medium and grown at 30°C for precisely 18 hours. Then, 50µl were transferred to 5ml LB and grown for 3 hours (precultures). The precultures were then diluted 1:200 (usually 50µl in 5ml medium) in bactopectone (with appropriate antibiotics, and for pHT304-pXyl constructs, xylose in appropriate concentrations (see results)).

The bactopectone cultures were then transferred to 96-well polyvinylchloride (PVC) plates, 125µl in each well. The plates were put in a moist compartment with a lid, and put inside an incubator on 30°C and 50 rpm. Two different incubators were tested, but with no significant differences. After growth (24, 48, 72 or 96 hours), the plates were washed and the cultures stained with crystal violet in the following manner:

- 1) Each well was washed with 130µl PBS
- 2) Cells in each well were stained with 130µl crystal violet (0,3%-1%) for 20 minutes
- 3) Each well were washed with 180µl PBS
- 4) To solubilise the bound, stained cells, 150µl 1:4 acetone/ethanol solution was added to each well

The absorbance of each well was then measured on 490nm with a HTS 7000 Bio Assay Reader (Perkin Elmer). The absorbance for crystal violet is optimal around 590nm, but the lab lacked appropriate filters for the assay reader.

## Chapter 5: Results

### 5.1 Bioinformatic analysis of Bt407\_0422 and Bt407\_1060

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As stated in background for the thesis (chapter 2), Bt407\_0422 and Bt407\_1060 are two genes located downstream of the Bc1 and Bc2 riboswitches originally identified by Sudarsan and co-workers [63] in *Bacillus cereus* ATCC 14579 and ATCC 10987, respectively. Initial analysis of the gene sequences asserted that the protein products may be important for chemotaxis and adhesion, two functions that are known to be important for biofilm formation. It was decided to expand the analysis further to ascertain 1) what domains the proteins contain, 2) where the domains are located in the protein sequence and 3) identify where the protein probably is situated in the bacteria. A limited BLAST search of the protein sequences was also done to get an indication on prevalence of resembling proteins in other fully, closed sequenced *Bacillus* strains. A list of the strains included in the BLAST search is given below:

- *Bacillus cereus* 03BB102
- *Bacillus cereus* AH187
- *Bacillus cereus* AH820
- *Bacillus cereus* ATCC 10987
- *Bacillus cereus* ATCC 14579
- *Bacillus cereus* B4264
- *Bacillus cereus* E33L
- *Bacillus cereus* G9842
- *Bacillus cereus* Q1
- *Bacillus anthracis* Ames
- *Bacillus anthracis* Ames Ancestor
- *Bacillus anthracis* Sterne
- *Bacillus anthracis* str. A0248
- *Bacillus anthracis* str. CDC 684
- *Bacillus cytotoxicus* NVH 391-98

- *Bacillus thuringiensis* serovar konkukian str. 97-27
- *Bacillus thuringiensis* str. Al Hakam
- *Bacillus thuringiensis* 407
- *Bacillus weihenstephanensis* KBAB4

### 5.1.1 Sequence analysis of Bt407\_1060

A BLAST search of the protein sequence (Appendix 7) of Bt407\_1060 was performed to investigate the prevalence of Bt407\_1060 in the *Bacillus cereus* group using the 19 closed genomes available in Genbank. The search resulted in several hits for various proteins with adhesive properties in members of the *B. cereus* group. Results from the search are summarized in table 5.1, showing that only 5 of 19 strains contained a designated ortholog (>90% similarity, full query length (2185/2185 amino acids)).

Strain (s)	Identity	Similarity	Query coverage
<i>Bacillus cereus</i> G9842	2111/2185 (96%)	2138/2185 (97%)	100%
<i>Bacillus weihenstephanensis</i> KBAB4	1936/2168 (89%)	2019/2168 (93%)	99%
<i>Bacillus cereus</i> ATCC 14579	1872/2062 (90%)	1949/2062 (94%)	100%
<i>Bacillus cereus</i> B4264	1805/2050 (88%)	1913/2050 (93%)	100%
<i>Bacillus thuringiensis</i> str. Al Hakam	1613/1773 (90%)	1672/1773 (94%)	100%

**Table 5.1: BLAST search results of the 19 closed genome sequences available from the *B. cereus* group bacteria, Bt407\_1060 protein sequence as query sequence.**

Homologous protein sequences found in other strains in the BLAST query contained <30% identity and 50% similarity to the original sequence. These results were not included since the intent of this search was to identify the extent of closely resembling genes in the *Bacillus cereus* group (orthologs). These paralogous gene sequences may certainly carry similar functions, but this is difficult to establish on the basis of this query alone.

An InterPro scan [1] of the Bt407\_1060 protein sequence revealed several key aspects which may be important for its function. Two domains resembling Cna protein B-type domain [83] and collagen binding domain [84], two domains previously identified in *Staphylococcus aureus* to be vital parts of the collagen-binding adhesion protein Cna located at the bacterial surface. The Cna protein B-type domain is necessary to elevate the ligand- binding domain, collagen binding domain, away from the cell membrane while the collagen binding domain contain adhesion properties.

This domain is part of the bacterial adhesins superfamily.

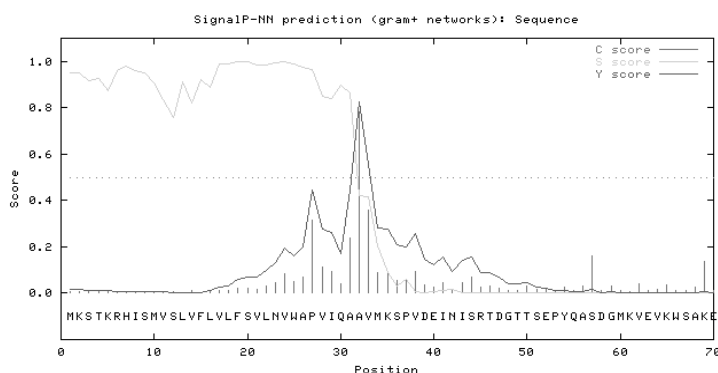
Furthermore, the InterPro scan also identified an LPXTG anchor in the C- terminal, which indicates that sortase or a related enzyme could potentially cleave between the amino acids



**Figure 11: Putative conserved domains in the protein sequence of Bt407\_1060 analyzed by InterProScan [1].**

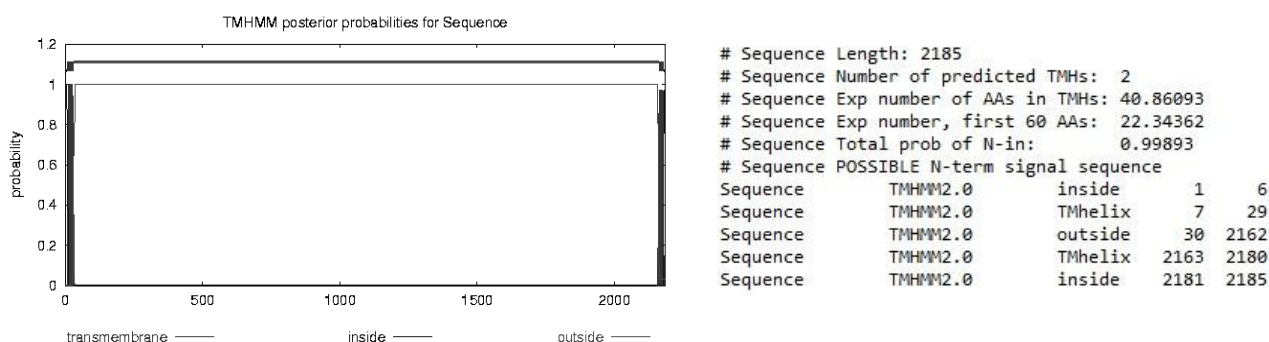
threonine and glycine in the region, which is known to result in a covalent anchoring of the C- terminal to the cell wall. An N- terminal signal sequence and transmembrane helix regions were also identified (figure 11).

SignalP [2] output revealed a signal peptide cleavage site, which is indicative of proteins transported out of the cell by the general secretion machinery, between amino acids 31 and 32 from the N- terminal (figure 12). A search for twin- arginine peptide cleavage sites (TaTP) turned out negative (not shown).



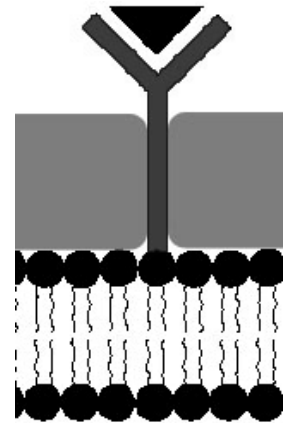
**Figure 12: SignalP output using the entire Bt407\_1060 protein sequence as query [2] showing a possible N- terminal signal sequence between amino acids 31 and 32.**

Transmembrane helix domains were identified in InterPro near both the N and C-terminal of the protein. TMHMM [3] output gave the same result (figure 13).



**Figure 13: Output from TMHMM analysis of the complete Bt407\_1060 protein sequence, indicating the presence of two transmembrane regions in the protein [3]**

On the basis of the accumulated data, it seems probable that Bt407\_1060 is a protein transported out of the bacteria, anchored to the cell wall extracellularly at the C- terminal and carry adhesion properties. The InterPro scan identify the sensory domains at the N-terminal while a repetitive domain make up the back bone of the protein, elevating the sensory areas of the protein away from the cell membrane of the bacteria (figure 14).



**Figure 14: Theoretical location of the Bt407\_1060 protein. The C-terminal is anchored to the cell wall and the rest of the protein is directed away from the cell.**

### 5.1.2 Sequence analysis of Bt407\_0422

A BLAST [85] search of the protein sequence (Appendix 7) was performed to investigate the prevalence of Bt407\_0422 in the *B. cereus* group, using the 19 closed genomes available in Genbank. The search resulted in several hits for various methyl-accepting chemotaxis protein(s) in members of the *Bacillus cereus* group. Results are summarized in table 5.2, showing that 4 of 19 strains contained a clear ortholog to Bt407\_0422 (99% identity, full query length (580/580 amino acids)), and that 15/19 strains contained a protein with >80% similarity in sequence.

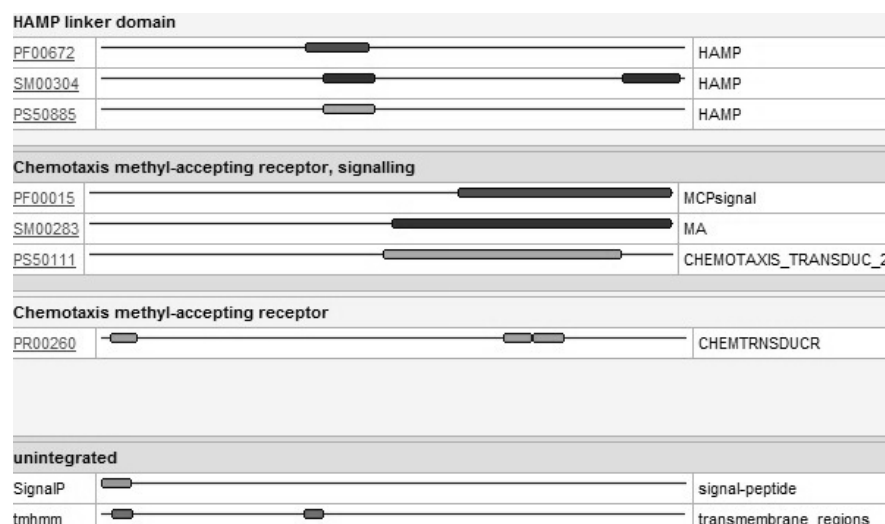
Homologous protein sequences were also found in other strains by the BLAST query, which contained <30% identity and 50% similarity to the original sequence. These results were not included since the focus was on identifying the extent of closely resembling genes in the *Bacillus cereus* group (orthologs). These paralogous gene sequences may certainly carry similar functions, but this is difficult to establish on the basis of this query alone.



Strain (s)	Identity	Similarity	Query coverage
<i>Bacillus thuringiensis</i> Bt407	580/580 (100%)	580/580 (100%)	100%
<i>Bacillus cereus</i> G9842	578/580 (99%)	579/580 (99%)	100%
<i>Bacillus cereus</i> ATCC 14579	576/580 (99%)	579/580 (99%)	100%
<i>Bacillus cereus</i> B4264	576/580 (99%)	579/580 (99%)	100%
<i>Bacillus weihenstephanensis</i> KBAB4	475/579 (82%)	524/579 (90%)	99%
<i>Bacillus cereus</i> NVH391-98 (“cytotoxicus”)	450/580 (77%)	515/580 (88%)	100%
<i>Bacillus thuringiensis</i> serovar konkukian Str. 97-27	445/579 (76%)	508/579 (87%)	99%
<i>Bacillus cereus</i> 03BB102	445/579 (76%)	508/579 (87%)	99%
<i>Bacillus cereus</i> AH820	444/579 (76%)	507/579 (87%)	99%
<i>Bacillus cereus</i> AH187	445/579 (76%)	504/579 (87%)	99%
<i>Bacillus cereus</i> Q1	445/579 (76%)	504/579 (87%)	99%
<i>Bacillus anthracis</i> str. Ames	443/579 (76%)	506/579 (87%)	99%
<i>Bacillus cereus</i> ATCC 10987	443/579 (76%)	503/579 (86%)	99%
<i>Bacillus thuringiensis</i> str. Al Hakam	440/580 (75%)	505/580 (87%)	99%
<i>Bacillus cereus</i> E33L	441/579 (76%)	503/579 (86%)	99%

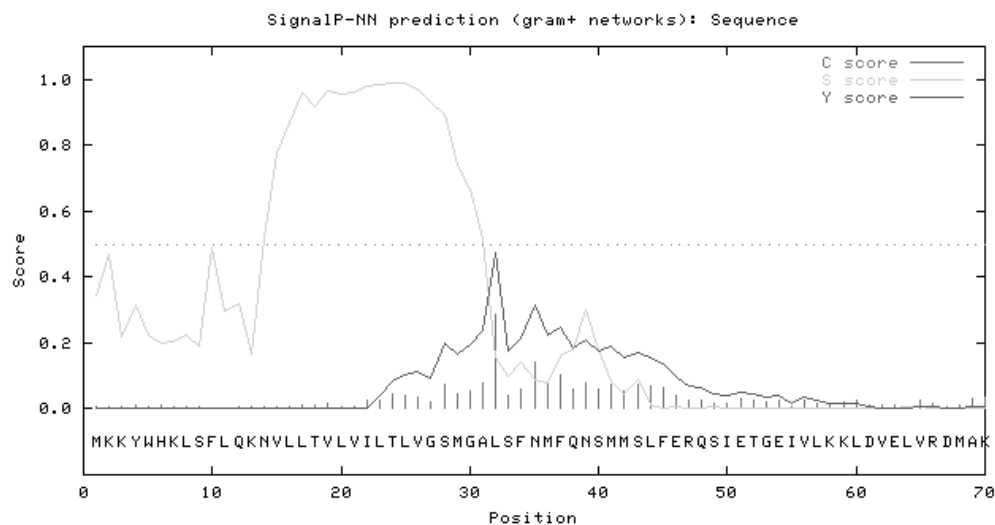
**Table 5.2: BLAST search results of the 19 closed genome sequences available from the *B. cereus* group bacteria, using the Bt407\_0422 protein sequence as query sequence.**

An InterPro [1] search was performed to identify characterized domains in the protein sequence, and showed that the Bt407\_0422 protein sequence carried domains known to be a part of various chemotaxis methyl- accepting proteins (MCP's)(figure 15). One such domain, a methyl- accepting chemotaxis protein (MCP) signalling domain found in *Bacillus subtilis*, has been shown to be homologous to CheW in *E. coli*, which is important for intracellular signalling resulting in flagellar rotation. A *B. subtilis* *cheW* mutant displayed impaired chemotaxis behaviour [86]. The HAMP linker domain is found in several bacterial sensor and chemotaxis proteins [87] and is important for conformational changes in the receptor when exposed to ligand [88].



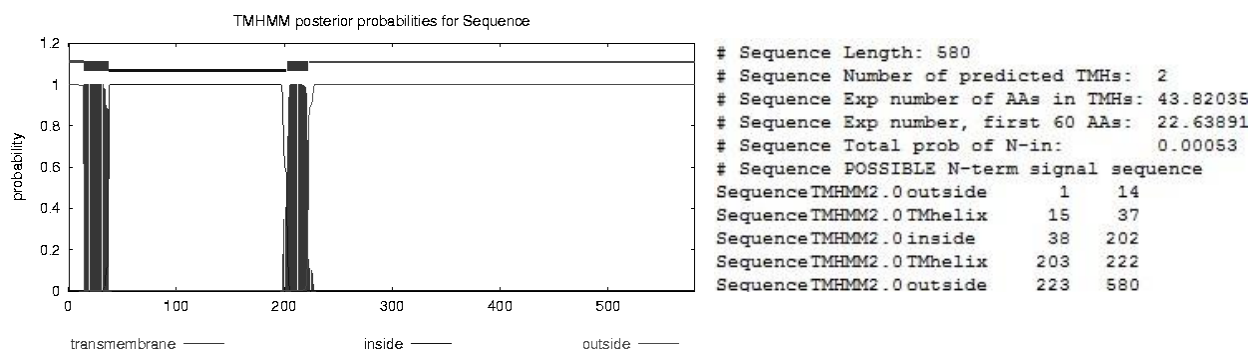
**Figure 15: Putative conserved domains in the protein sequence of Bt407\_0422 analyzed by InterProScan [1].**

The InterPro scan also revealed a possibility that the Bt407\_0422 protein sequence contained a SignalP domain, a signal peptide cleavage site which is indicative of proteins transported out of the cell by the general secretion machinery. A SignalP [2] search confirmed the most likely cleavage site between amino acid 30 and 31 from the N- terminal (figure 16). A search for twin-arginine signal peptide cleavage sites common in bacteria [89] did not produce any positive hits (not shown).



**Figure 16: SignalP output using the entire Bt407\_0422 protein sequence as query [2] showing a possible N- terminal signal sequence between amino acids 30 and 31.**

In order to investigate for transmembrane helices in Bt407\_0422, a TMHMM [3] search of the protein sequence was done (figure 17).

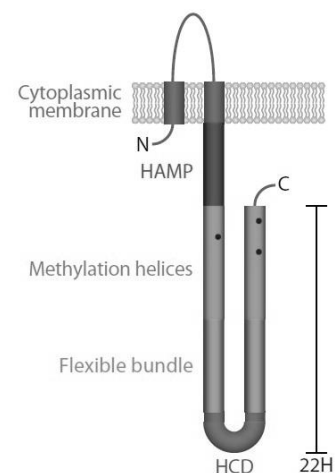


**Figure 17: Output from TMHMM analysis of the complete Bt407\_0422 protein sequence, indicating the presence of two transmembrane regions in the protein [3]**

The TMHMM output indicated two transmembrane regions with a large intracellular part. The output does not correlate with the general model for methyl- accepting chemotaxis proteins (MCP's, figure 18), where the general notion is that the N- terminal is on the cytoplasmic side, followed by a cytosolic region where ligands may be anchored and a cytoplasmic region containing the HAMP domain and other domains necessary for intracellular signalling [4].

However, the TMHMM software is prone to not identify intracellular and extracellular regions correctly, and thus the knowledge of two membrane- spanning regions is valuable.

On the basis of the accumulated data, it seems probable that the Bt407\_0422 protein is a methyl- accepting chemotaxis protein and several orthologs exists in various strains belonging to the *Bacillus cereus* group. The presence of the signalP cleavage site indicates that the Bt407\_0422 protein is probably transported by the general secretion system out of the cell where it is located in the membrane, containing two membrane- spanning regions. This correlates well with the domains found in the InterPro scan, the designated extracellular region may contain sensory functions while the intracellular region contain HAMP and other signalling domains.



**Figure 18: General model for methyl-accepting chemotaxis proteins in bacteria [4]**

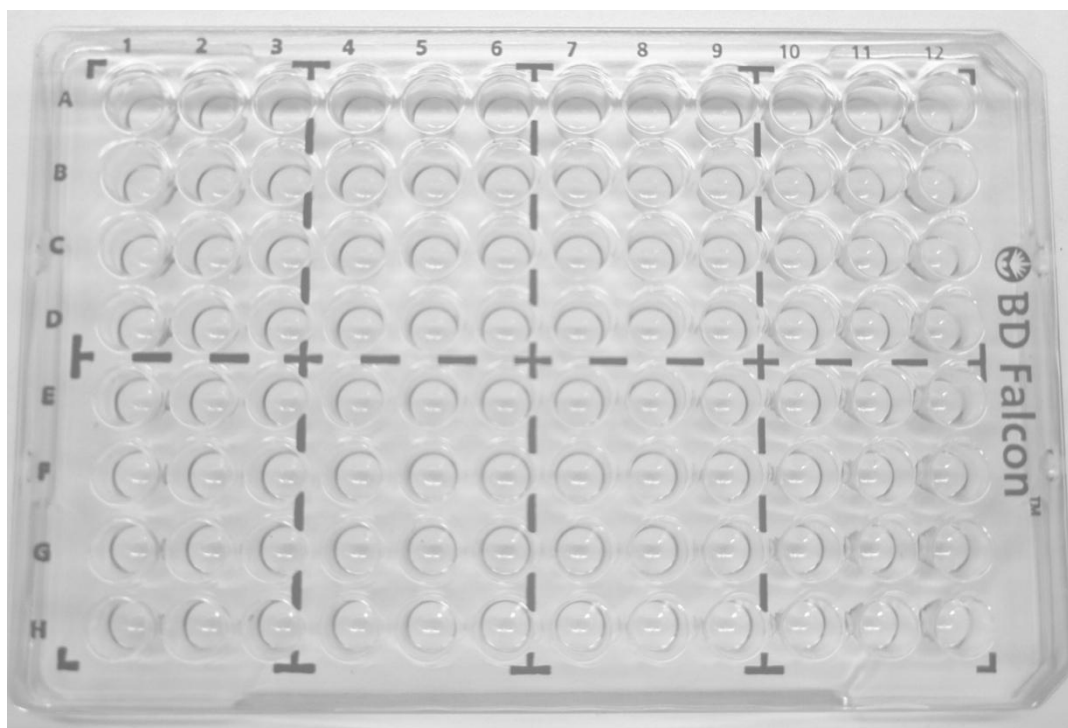
## 5.2 Biofilm screening of *Bacillus thuringiensis* wt and its isogenic gene deletion strains $\Delta$ Bt407\_0422 and $\Delta$ Bt407\_1060

The first biofilm assay was done to screen for the efficiency of biofilm formation from wt and its isogenic gene deletion strains  $\Delta$ Bt407\_0422 and  $\Delta$ Bt407\_1060 in *B. thuringiensis* 407. The two isogenic gene deletion strains were already prepared by Annette Fagerlund

prior to the thesis, and a small scale microtiter plate assay had been done where it was indicated that  $\Delta Bt407\_1060$ - mutant produce less biofilm compared to wildtype, and the  $\Delta Bt407\_0422$  mutant produce less biofilm during the first 24 hours compared to wildtype, but no difference after 48 and 72 hours.

### 5.2.1 Microtiter plate assay

In order to screen several *Bacillus* strains under identical conditions, a microtiter plate assay was chosen. The microtiter plate assay is useful for the study of biofilm formed at the air-liquid interface, and it is easy to carry out high- throughput screening of several strains at the same time. General protocol (4.13) had been established prior to this thesis by collaboration with Michel Gohar, INRA, France, and has been used for earlier research on biofilm in *Bacillus cereus* [34, 90]. The protocol was tested and put to practical use in the lab by a former master student, Johan Bjørnstad.



**Figure 19: Falcon 353011 96- wells PVC microtiter plate used in the microtiter plate assay.**

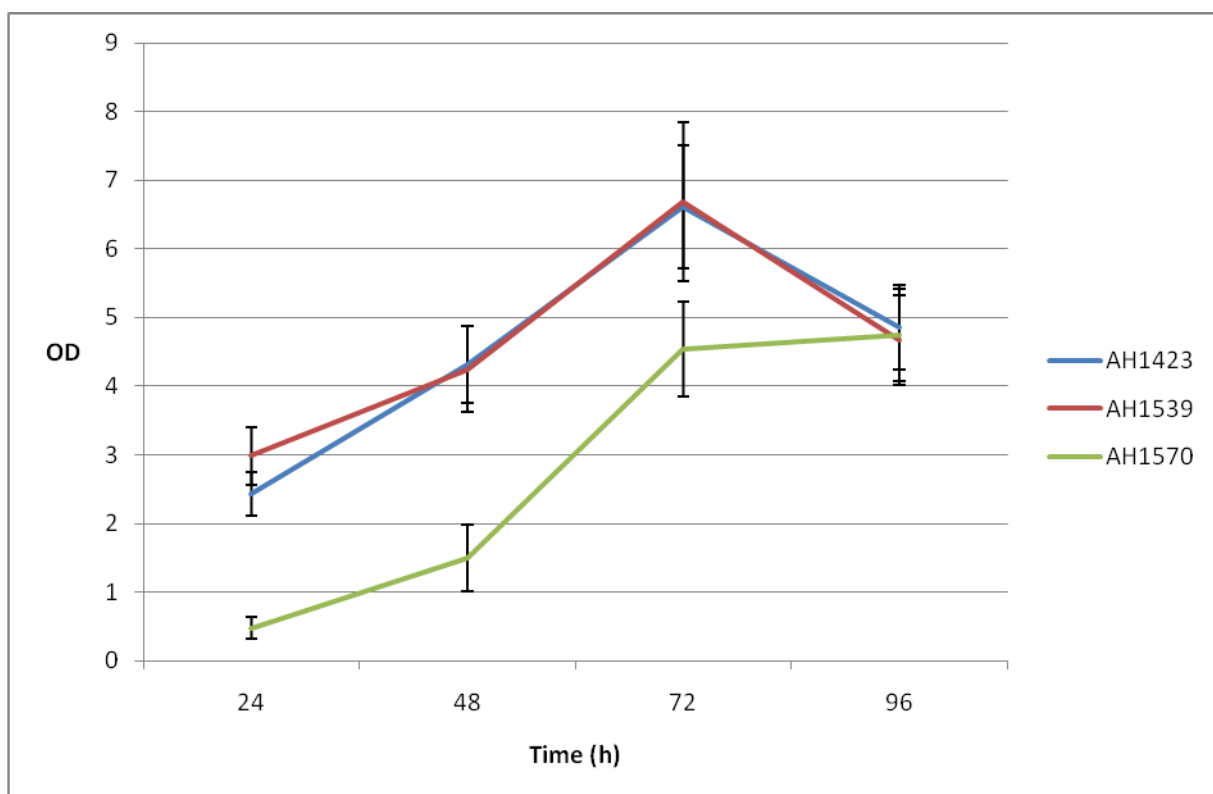
### 5.2.2 Biofilm screening of Bt 407 wt, $\Delta$ Bt407\_0422 and $\Delta$ Bt407\_1060 strains

*B. thuringiensis* 407 wildtype,  $\Delta$ Bt407\_0422 and  $\Delta$ Bt407\_1060, were grown and prepared according to protocol (4.13). The setup of the plates (table 5.3) was designed to get 16 replicates for each strain on every plate. The design with bactopectone in between the lanes containing a *B. thuringiensis* 407 strain was done to control whether application of bacteria would spread from the designated well to wells adjacent to it, and practical control of the researcher. Eight identical plates were prepared in order to get two replicates for every time interval, 24h, 48h, 72h and 96h

	1	2	3	4	5	6	7	8	9	10	11	12
A	AH1423 (wt)	Bactopeptone (blank)	AH1539 ( $\Delta$ 0422)	Bactopeptone (blank)	AH1570 ( $\Delta$ 1060)	Bactopeptone (blank)	AH1423 (wt)	Bactopeptone (blank)	AH1539 ( $\Delta$ 0422)	Bactopeptone (blank)	AH1570 ( $\Delta$ 1060)	Bactopeptone (blank)
B												
C												
D												
E												
F												
G												
H												

**Table 5.3: Design of microtiter plate assay for surface biofilm formation for *B. thuringiensis* 407 and its isogenic gene deletion mutants  $\Delta$ Bt407\_0422 and  $\Delta$ Bt407\_1060.**

The first biofilm assay revealed problems with the initial protocol. The HTS 7000 Bio Assay Reader used for OD measurements had a cutoff at OD 3. OD measurements of wt and  $\Delta 0422$  strains after 48h and 72h exceeded the cutoff of the assay reader and every well had to be diluted 1:2.5 (50 $\mu$ l:125 $\mu$ l) in order to get a proper reading. Diluting the wells after solubilization with acetone/ethanol was performed quickly in order to avoid extensive vaporization. Following crystal violet staining of the biofilm structures, the degree of biofilm formation was measured by optical density measurements (4.13). Results are shown in figure 20.



**Figure 20: Microtiter plate biofilm assay, three different strains, AH1423 (Bt407 wildtype), AH1539 ( $\Delta$ Bt407\_0422) and AH1570 ( $\Delta$ Bt407\_1060) were grown in 32 wells each on two sets of microtiter plates for 24, 48, 72 and 96 hours. The wells were washed, stained with 1% crystal violet, solubilized with acetone/ethanol and optical density (OD) was measured at 492nm. Vertical bars indicate standard deviation from  $\mu$ .**

This experiment showed significantly reduced biofilm formation by the  $\Delta$ Bt407\_1060 mutant compared to wildtype after 24, 48 and 72 hours of incubation which is a strong indicator that Bt407\_1060 is important for biofilm formation in *B. thuringiensis* 407. However, the experiment did not reveal any significant difference in biofilm formation by the  $\Delta$ Bt407\_0422 mutant compared to wildtype, which is a difference from the previous, smaller study done by Annette Fagerlund where a small difference in biofilm formation could be seen after 24 hours.

## 5.3 Preparation of electrocompetent cells

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In order to study complementation of Bt407\_0422 and Bt407\_1060 in the isogenic gene deletion mutants *Bacillus thuringiensis* 407  $\Delta$ Bt407\_0422 and  $\Delta$ Bt407\_1060, as well as overexpression studies in *B. thuringiensis* 407 wildtype, it was necessary to prepare constructs where these two genes were inserted into expression vectors specially made for cloning in *Bacillus* strains, pHT304-pXyl and pHT304-18z. These vectors are constructed to express ampicillin resistance in both *E. coli* and *Bacillus cereus*, enabling the researcher to do the preliminary cloning work done in *E. coli* prior to transformation into *B. cereus*. This is an advantage since cloning and commercial kits are readily available for *E. coli*, whereas cloning work in *B. cereus* are more complicated (further information concerning these vectors, see 5.4.3 and 5.6.3). In order to perform electrotransformation of plasmid DNA in the cloning procedure, it was necessary to prepare electrocompetent cells of *E. coli*.

Electrocompetent *E. coli*, using the strain *Escherichia coli* (*E. coli*) XL-1 Blue, were made following lab protocol (4.2.1). After washing and storage at  $-80^{\circ}\text{C}$ ,  $\sim 40\text{--}50\mu\text{l}$  cells from the new batch, as well as commercially prepared cells (TOP10, 3.1), were transformed with 10pg of three different plasmids, pUC19, pMAD and pHT304-18z (3.1.2), 1000 $\mu\text{l}$  SOC medium was added and the cells were incubated at  $37^{\circ}\text{C}$  for one hour. 1 $\mu\text{l}$ , 10 $\mu\text{l}$  and 100 $\mu\text{l}$  cell-medium suspension were plated out in duplicate LB plates with 100 $\mu\text{g}/\text{ml}$  ampicillin, and incubated at  $37^{\circ}\text{C}$  overnight. The cells were then counted using a colony counter (Gerber



Instruments), and colony forming units (CFU)/ $\mu\text{g}$  transformed DNA was estimated for the different plasmids. In order to estimate CFU/ $\mu\text{g}$  DNA, the mean of the three different plates (duplicate) were calculated and corrected for dilution. E.g. if 1 colony appeared on the LB plate after applying 1  $\mu\text{l}$  medium (1000  $\mu\text{l}$ =10pg):

$$\frac{1 \text{ CFU}}{\frac{1}{1000} 10 \text{ pg DNA}} = \frac{1 \text{ CFU}}{0,01 \text{ pg}} = \frac{100 \times 10^6 \text{ CFU}}{\mu\text{g}} = 1 \times 10^8 \text{ CFU}/\mu\text{g}$$

CFU/ $\mu\text{g}$  for each plate was then estimated, and an overall mean calculated for each plasmid:

pUC19	CFU electrocompetent cells
1 $\mu\text{l}$ medium	18,5
10 $\mu\text{l}$ medium	372,5
100 $\mu\text{l}$ medium	2543
<b>Mean CFU/<math>\mu\text{g}</math> DNA</b>	$2,706 \times 10^9 \text{ CFU}/\mu\text{g}$

**Table 5.4: Estimation of CFU/ $\mu\text{g}$  DNA after transformation of 10pg pUC19 plasmid into *E. coli* and corrected for dilution (see above for calculation).**

pMAD	CFU electrocompetent cells
1 $\mu\text{l}$ medium	5
10 $\mu\text{l}$ medium	47
100 $\mu\text{l}$ medium	540
<b>Mean CFU/<math>\mu\text{g}</math> DNA</b>	$5,033 \times 10^8 \text{ CFU}/\mu\text{g}$

**Table 5.5: Estimation of CFU/ $\mu\text{g}$  DNA after transformation of 10pg pMAD plasmid into *E. coli* and corrected for dilution (see above for calculation).**

<b>pHT304-18z</b>	<b>CFU electrocompetent cells</b>
1µl medium	2
10µl medium	34
100µl medium	512
<b>Mean CFU/µg DNA</b>	$4,26 \times 10^8 \text{ CFU/µg}$

**Table 5.6: Estimation of CFU/µg DNA after transformation of 10pg pHT304-18z plasmid into *E. coli* and corrected for dilution (see above for calculation).**

<b>pHT304-pXyl</b>	<b>CFU electrocompetent cells</b>
1µl medium	12
10µl medium	103
100µl medium	972
<b>Mean CFU/µg DNA</b>	$9,985 \times 10^8 \text{ CFU/µg}$

**Table 5.7: Estimation of CFU/µg DNA after transformation of 10pg pHT304-pXyl plasmid into *E. coli* and corrected for dilution (see above for calculation).**

The estimated CFU/µg for this batch was satisfactory, and the cells were expected to work well during the following cloning procedures. The results indicated that the transformation efficiency of the different plasmids varied, pUC19 (table 5.4) was the most efficient plasmid, and vastly more efficient compared to the vector pMAD (table 5.5).

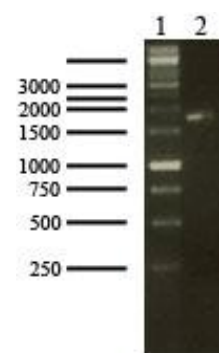
## 5.4 Cloning of Bt407\_0422 into pHT304-pXyl reporter vector

In order to study complementation of Bt407\_0422 in *Bacillus thuringiensis* 407  $\Delta$ Bt407\_0422 and overexpression of Bt407\_0422 in *B. thuringiensis* 407 wt, it was decided that the gene Bt407\_0422 first would be cloned in a TOPO II vector and transferred, after proper verification of correct sequence, into the expression vector pHT304-pXyl. The Bt407\_0422 – pHT304-pXyl construct would then be transferred to the various strains of *B. thuringiensis* 407 for the functional studies concerning biofilm formation.

The pHT304-pXyl vector is a shuttle vector constructed for cloning in *B. thuringiensis* and *E. coli*; it contains two antibiotic resistance markers, an ampicillin resistance gene functional in *E. coli* and an erythromycin resistance gene functional in both *E. coli* and *B. thuringiensis* [71]. Additionally, this particular vector contains the *xylA* promoter from *Bacillus subtilis*, which is induced by increasing xylose concentrations, reaching a plateau at 16,7mM [91].

### 5.4.1 PCR

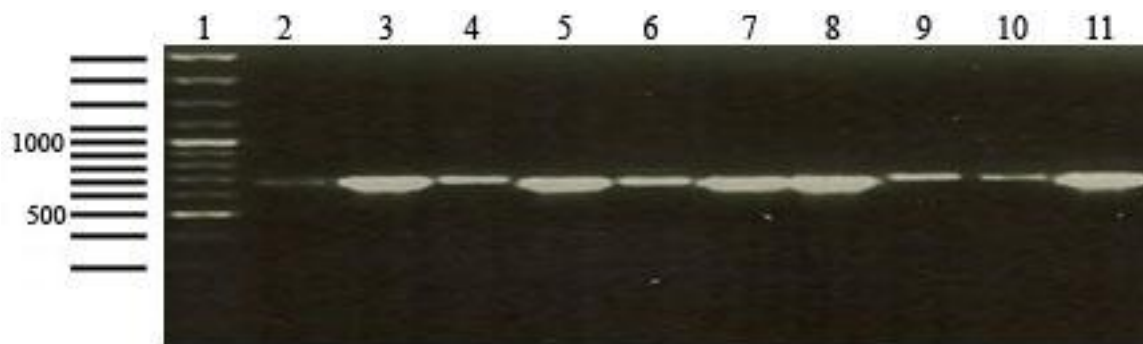
Initial PCR (4.5) amplification of the Bt407\_0422 gene was performed with primers Bt407\_0422-F-XbaI-/Bt407\_0422-R-KpnI (3.3), which would initiate replication upstream and downstream of the gene Bt407\_0422 in the *B. thuringiensis* 407 genome. A genome prep made from *B. thuringiensis* 407 was used as template. The length of the PCR product was expected to be ~1816bp. 10 $\mu$ l of the PCR product was applied to a gel (4.6) for control, along with a negative control lacking template DNA. The PCR product was of the expected size (figure 21).



**Figure 21:** Lanes: 1: 1kb st. (Fermentas), 2: PCR product Bt407\_0422 . Lane 3: Negative control without genome as template, no bands (not shown)

### 5.4.2 TOPO cloning

For the next step, it was desirable to clone the Bt407\_0422 PCR product into a TOPO vector to be able to do qualitative control of the Bt407\_0422 fragment prior to transfer into the shuttle vector pHT304-pXyl. The PCR product was inserted into a TOPO II vector following standard protocol (4.12). The Bt407\_0422 – pHT304-pXyl construct was then transformed into electrocompetent *E. coli* XL-1 blue MRF- cells (4.11.1) and incubated on LB plates containing 100 µg /ml ampicillin / 50 µg/ml kanamycin / 80µg/ml x-gal. Ten white colonies were picked and controlled using colony PCR (4.5) with primers S2\_pUC19R/00330-Bt407\_0422-R2 (3.3) and controlled by agarose electrophoresis for size and specificity (4.6). The expected size of the PCR product was ~673bp.

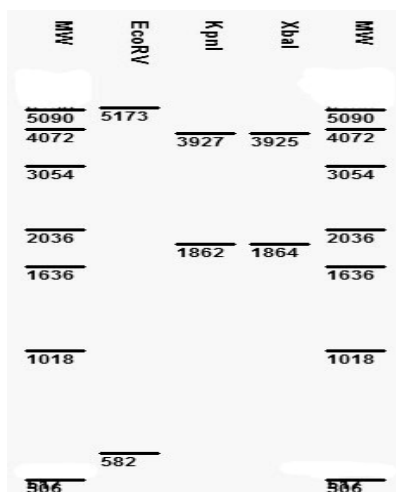


**Figure 22: Colony PCR of the Bt407\_0422 – TOPO construct. Lanes: 1: 100bp st. (Fermentas), lanes 2-11: PCR products from colony 1-10 picked from LB plates.**

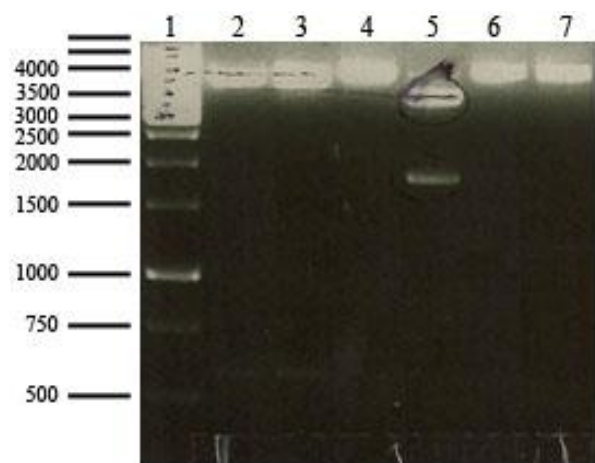
Electrophoresis showed that nearly all colonies seemed to contain the desired PCR product - TOPO plasmid (figure 22), but it was speculated that some of the wells were overflowed after application of reactions on the gel. Two colonies were prepared for miniprep and restriction testing, while four other were prepared for glycerol stocks.

### 5.4.3 Restriction testing

Plasmid minipreps were prepared (4.3.2) from the two colonies from lane 3 and 7, and were tested by restriction digestion using enzymes *EcoRV* and *KpnI* (4.7). The colony from lane 7 had expected sizes after analysis by agarose electrophoresis (4.6)(figure 23 and 24) whereas the colony from lane 3 lacked the *KpnI* restriction site.



**Figure 23: *In silico* restriction test, expected band size with *EcoRV*, *KpnI* and *XbaI* restriction enzymes**

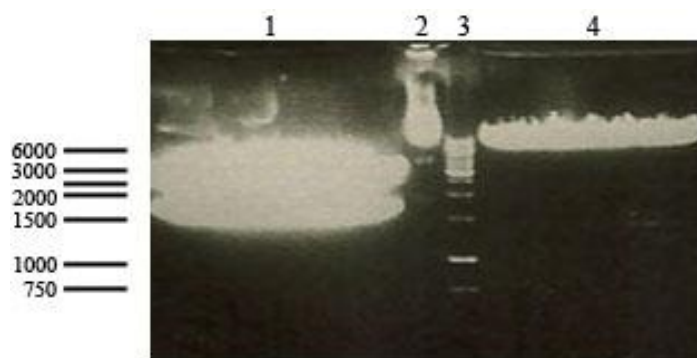


**Figure 24: Restriction test of 2 colonies containing Bt407\_0422- TOPO. Lanes: 1: 1kb st. (Fermentas), 2: Colony 1 cut with *EcoRV*, 3: Colony 2 cut with *EcoRV*, 4: Colony 1 cut with *KpnI*, 5: Colony 2 cut with *KpnI*, 6-7: Colony 1 and 2 without restriction enzymes.**

It was decided to proceed with a large digest and subcloning the Bt407\_0422 insert from the colony from lane 7 into the pHT304-pXyl expression vector.

#### 5.4.4 Subcloning of Bt407\_0422 in pHT304-pXyl vector

In order to express Bt407\_0422 in *B. thuringiensis* 407, we wished to move the cloned Bt407\_0422 fragment from the Bt407\_0422 – TOPO vector to pHT304-pXyl. Bt407\_0422-TOPO and pHT304-pXyl were both double digested with *Xba*I and *Kpn*I and separated on a preparative agarose gel (4.6). The *Xba*I/*Kpn*I double digest had produced DNA bands of the expected sizes (figure 25).

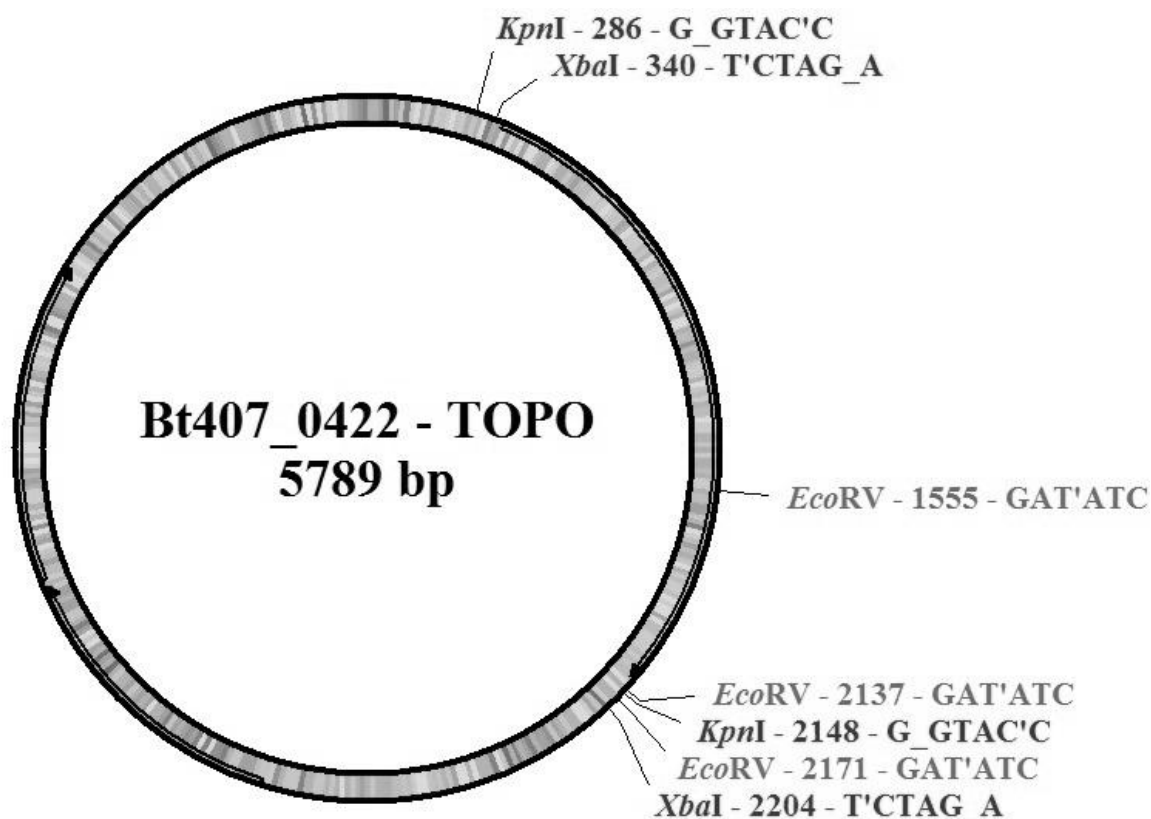


**Figure 25: Large restriction digest. Lanes: 1: Bt407\_0422 –TOPO double digested with *Xba*I and *Kpn*I, 2: Uncut pHT304-pXyl vector, 3: 1kb st. (Fermentas), 4: pHT304-pXyl double digested with *Xba*I and *Kpn*I. The Bt407\_0422 band can be seen on the left (~2000bp) and was excised. It was separated from the TOPO vector and uncut vector (~4000-6000bp). On the right, the pHT304-pXyl vector is separated from uncut vector (top band on the right) and was excised.**

As can be seen in figure 25, the Bt407\_0422 fragment (~2000bp) was separated from the TOPO vector and uncut vector, and was excised carefully from the gel. The digested pHT304-pXyl vector (clearly visible band with ~8000bp) was separated from uncut vector (top band) and was also excised from the gel. Both DNA fragments were purified by using a commercial PCR purification kit (4.3.1) and concentrations of DNA was measured using both spectrophotometry (4.4.1) and quantitative gel electrophoresis (4.4.2).

The next step was to ligate the digested Bt407\_0422 insert with the digested pHT304-pXyl vector. Two reactions were set up, one reaction with 1:3 molar ratio of vector/insert and one reaction with 1:5 molar ratio, and ligated overnight at 16°C (4.8). Ligation reactions were then dialyzed on filter paper for 30 minutes, transformed into electrocompetent *E. coli* (4.11.1) and plated out on LB plates containing 100µg/ml ampicillin / 200µg/ml erythromycin. No colonies were detected after 24 hours of growth at 37°C. A second identical cloning experiment did not produce any colonies.

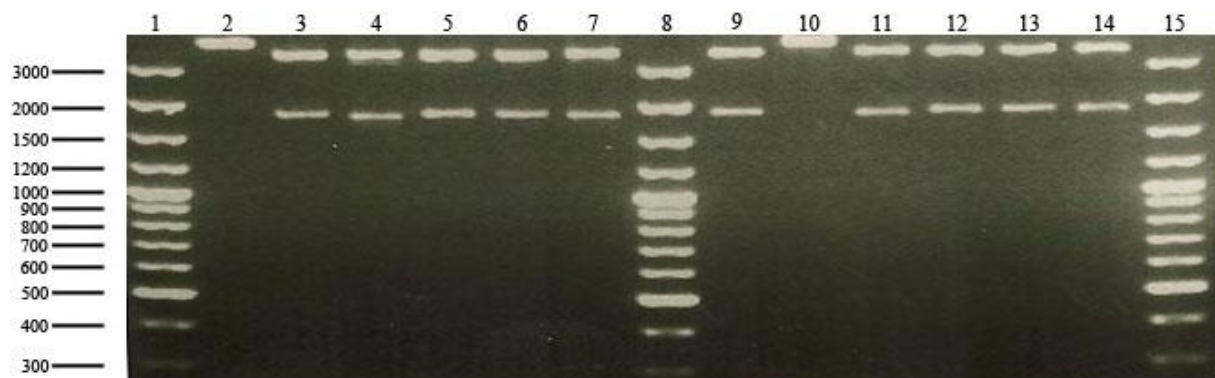
It was discovered that the Bt407\_0422-TOPO construct did not contain the restriction site for *Xba*I when the Bt407\_0422 – TOPO vector was tested with *Xba*I and *Kpn*I separately. The reason why this was not discovered earlier was that the BC0422- TOPO vector contains two restriction sites for both *Xba*I and *Kpn*I (figure 26) which are located close to each other. When *Xba*I was not present, the fragment produced by digest with *Kpn*I would still be approximately the same length and, therefore, not possible to discover with a double digest.



**Figure 26: Bt407\_0422 – TOPO vector with restriction sites marked. The Bt407\_0422 insert is located from the *Xba*I site (340) to the *Kpn*I site (2148).**

Unfortunately, the original restriction test did not include *Xba*I, only *Kpn*I and *Eco*RV. Restriction tests with *Xba*I and *Kpn*I on minipreps from the four untested, positive colonies (5.3.3) turned out to be negative. It was decided to start the cloning procedure with a new PCR amplification of the Bt407\_0422 gene in *B. thuringiensis* 407.

Cloning of Bt407\_0422 PCR product and insertion into TOPO vector was done as previously described (5.4.1-5.4.3). Minipreps from four colonies supposed to contain Bt407\_0422 – TOPO was prepared (4.3.2) and restriction tested with *Xba*I, *Kpn*I and *Eco*RI (figure 27). Two of the colonies turned out to have both restriction sites intact, and the DNA fragments had the correct size. It was decided to proceed with colony 4.



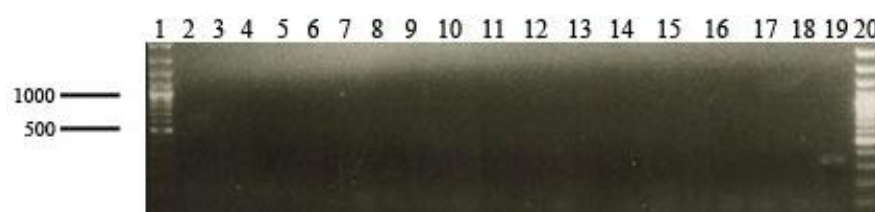
**Figure 27: Restriction test of four colonies supposed to contain Bt407\_0422 – TOPO. Lanes: 1: 100bp st. (Fermentas), 2-4: Colony 1 cut with *Xba*I, *Kpn*I and *Eco*RI, 5-7: Colony 2 with *Xba*I, *Kpn*I and *Eco*RI, 8: 100bp st. (Fermentas), 9-11: Colony 3 cut with *Xba*I, *Kpn*I and *Eco*RI, 12-14: Colony 4 cut with *Xba*I, *Kpn*I and *Eco*RI, 15: 100bp st. (Fermentas)**

Two new attempts at ligating the Bt407\_0422 fragment with the pHT304-pXyl vector did not give any colonies (not shown). Visual inspection of the LB plates after ligating the fragments, transformation into *E. coli* and 24 hours of growth at 37°C showed a viscous layer of cell debris. It was attempted to streak samples of this layer onto fresh LB plates containing 100µg/ml ampicillin / 200µg/ml erythromycin, but no colonies were detected. It was then discovered by comparing protocols in the lab that the erythromycin concentration for growth



of *E. coli* containing pHT304-pXyl constructs preferably should've been 400µg/ml, not 200µg/ml.

A fourth attempt resulted in several colonies (>100) on the plates after 24 hours of growth. Colony PCR with primers pHT304-pXyl\_forward/00330-Bt407\_0422-R2, which would amplify a part of the pHT304-pXyl vector and a part of the Bt407\_0422 fragment (expected size 780bp), turned out negative for 19 colonies. A positive control with an empty vector turned out to be positive (figure 28).



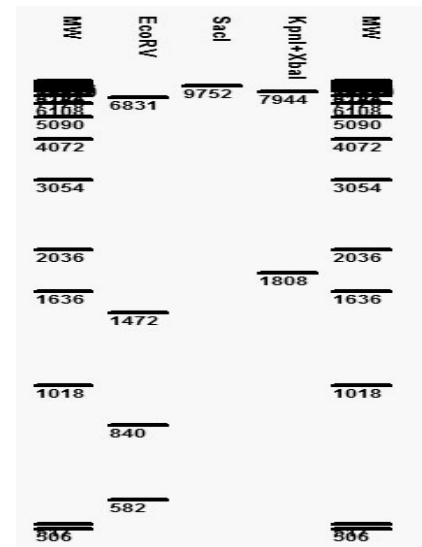
**Figure 28: Colony PCR of colonies supposed to contain Bt407\_0422 – pHT304-pXyl.**  
**Lanes: 1: 100bp st. (Fermentas), 2-18: Colony 1-18, 19: Positive control with empty vector (pHT304-pXyl), 20: 100bp st. (Fermentas)**

In order to understand what plasmid was present in the colonies that appeared on the plates, several tests were done. A miniprep was prepared for one colony (4.3.2) and tested with *XbaI* and *KpnI*, but no DNA bands were detected after gel electrophoresis (4.6, not shown).

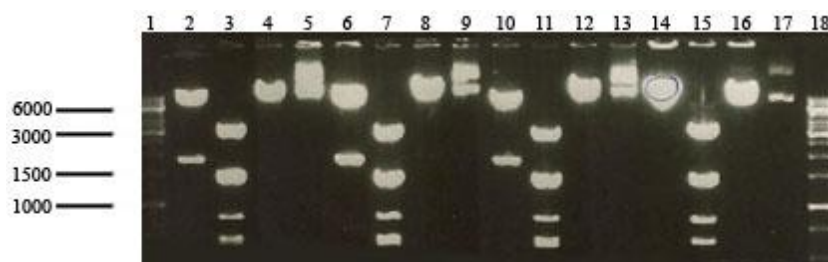
A colony PCR with three different primer sets (pHT304-pXyl\_forward/S2\_pUC19R, S1\_pUC19F/S2\_pUC19R and Bt407\_0422-*XbaI*-forward/00330-Bt407\_0422-R2) turned out negative (not shown).

However, an identical cloning procedure done by Annette Fagerlund gave several colonies. It was discovered that colonies would develop both after 24 and 48 hours of growth, all colonies tested previously was picked after 24 hours. In this cloning experiment, colonies were counted after 24 hours and 48 hours, and 40 colonies that appeared after 48 hours were tested by colony PCR with primers pHT304-pXyl\_forward/00330-Bt407\_0422-R2.

The colony PCR, done by Annette Fagerlund, showed that >20 colonies contained the Bt407\_0422- pHT304- pXyl construct (not shown). It was decided to proceed with four colonies. Minipreps were made (4.3.2), and each plasmid prep was restriction tested with *XbaI-KpnI* (double digest), *EcoRV*, *SacI* and a non-digested miniprep to be able to separate digested and non- digested DNA (figure 29 and 30). All four colonies had the expected number and sizes of bands.



**Figure 29: *In silico* restriction test, expected band sizes after digest with 3 enzymes in Bt407\_0422-pHT304-pXyl construct**



**Figure 30: Restriction test of four colonies supposed to contain Bt407\_0422 – pHT304-pXyl. Lanes: 1: 1kb st. (Fermentas), 2-5: Colony 1 cut with *KpnI-XbaI*, *EcoRV*, *SacI* and uncut, 6-9: Colony 2 with *KpnI-XbaI*, *EcoRV*, *SacI* and uncut, 10-13: Colony 3 cut with *KpnI-XbaI*, *EcoRV*, *SacI* and uncut, 14-17: Colony 4 cut with *KpnI-XbaI*, *EcoRV*, *SacI* and uncut, 18: 1kb st. (Fermentas).**

### 5.4.5 Sequence analysis

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It was decided to proceed with colony 1. Plasmid DNA was prepared for sequencing (4.10) with primers pHT304-pXyl\_forward, S2\_pUC10R, 00330-Bt407\_0422-F2 and 00330-Bt407\_0422\_R2. The first and second primers are located upstream and downstream of the Bt407\_0422 insert while the third and fourth primers are located inside the Bt407\_0422 fragment, with opposite directions. Analysis (Appendix 4) revealed two mutations in the Bt407\_0422 sequence compared to the published sequence in Genbank. Since the cloning procedure up to this point had been difficult, it was decided to apply in vitro mutagenesis (4.9) to correct the mutations.

### 5.4.6 First mutagenesis

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For the first mutation, a miniprep (4.3.2) of Bt407\_0422-pHT304-pXyl was prepared. Primer set Bt407\_0422\_mut1\_F/Bt407\_0422\_mut1\_R (3.3) was used in the first mutagenesis. Following the Quikchange II protocol (4.9.2), 4µl of the sample and control (reaction identical to sample except that no primers are added) reaction, respectively, were transformed into electrocompetent *E. coli* XL-1 blue MRF cells (4.11.1) and plated on LB plates containing 100µg/ml ampicillin / 400µg/ml erythromycin. After incubation for 24 hours at 37°C, sample and control plates were counted. There were ~5x more colonies on the sample plates compared to control. This is a control of whether the restriction enzyme *DpnI* successfully digested the plasmids replicated in *E. coli* while leaving the newly synthesized plasmids from the PCR intact. Six colonies were picked and minipreps prepared (4.3.2). Samples from the six minipreps were sent for DNA sequencing with S2\_pUC19R as primer. This primer is located downstream of the Bt407\_0422 gene in the Bt407\_0422 – pHT304-pXyl vector. Analysis (Appendix 5) showed that the mutation had been corrected for colony 3, 4 and 6.

### 5.4.7 Second mutagenesis

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For the second mutation, a miniprep of Bt407\_0422- pHT304-pXyl from colony 3 from the first mutagenesis (5.4.6) was prepared. Primer set Bt407\_0422\_mut2\_F/Bt407\_0422\_mut2\_R (3.3) was used in the second mutagenesis. Following the Quikchange II protocol (4.9.2), 4µl of the sample and control reaction (reaction identical to sample except that no primers are added) were transformed into electrocompetent *E. coli* XL-1 blue MRF cells (4.11.1) and plated out on LB plates with 100µg/ml ampicillin/400µg/ml erythromycin. After incubation for 24 hours at 37°C, sample and control plates were counted. The amount of colonies on the sample and control plates was ~equal. This is a control of whether the restriction enzyme *DpnI* successfully digested the plasmids replicated in *E. coli* while leaving the newly synthesized plasmids from the PCR intact. In this case, the procedure had seemingly not been able to eradicate the original, mutated vector. Minipreps from four colonies were prepared and sent to sequence with S2\_pUC19R as primer. This primer is located downstream of the Bt407\_0422 gene in the Bt407\_0422 – pHT304-pXyl vector. Analysis (Appendix 6) of the four sequences showed that colony 2 and 4 no longer contained any of the two original mutations (5.4.5). Colony 2 from this procedure was chosen for further work. Bt407\_0422-pHT304-pXyl from this colony was transformed into electrocompetent *E. coli* XL-1 Blue MRF (4.11.1), and glycerol stocks were prepared and stored at -80°C (registered as AH1661 in the AH registry).

## **5.5 Complementation of *Bacillus thuringiensis* 407 $\Delta$ Bt407\_0422 and $\Delta$ Bt407\_1060 gene deletion mutants**

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Biofilm assays done with *Bacillus thuringiensis* 407  $\Delta$ Bt407\_0422 and  $\Delta$ Bt407\_1060 isogenic gene deletion mutants and wild type strains had previously shown that the deletion of Bt407\_1060 seemingly resulted in impaired biofilm formation (5.2).

In order to determine the role of Bt407\_0422 and Bt407\_1060 in biofilm formation in *B. thuringiensis* further, constructs of Bt407\_0422-pHT304-pXyl and Bt407\_1060-pHT304-pXyl were transformed into wildtype (wt) and gene deletion mutant strains of Bt 407 for over-expression and complementation studies on biofilm formation over time using the microtiter plate assay (4.13).

### **5.5.1 *B. thuringiensis* 407 (Bt 407) variant strains**

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In order to do complementary/over-expression studies, 10 variants of the *B. thuringiensis* 407 (Bt 407) strain were needed.

Name	AH register	Vector carried	Gene deletion	Use
Bt 407	AH1423	None	None	Wt
Bt 407 + pXyl	AH1684	pHT304-pXyl	None	Control
Bt 407 + Bt407_0422	AH1685	Bt407_0422- pHT304-pXyl	None	Bt407_0422 overexpression
Bt 407 + Bt407_1060	AH1686	Bt407_1060- pHT304-pXyl	None	Bt407_1060 overexpression
Bt 407 $\Delta$ 0422	AH1539	None	Bt407_0422	$\Delta$ Bt407_0422
Bt 407 $\Delta$ 0422 + pXyl	AH1688	pHT304-pXyl	Bt407_0422	Control
Bt 407 $\Delta$ 0422 + 0422	AH1687	Bt407_0422- pHT304-pXyl	Bt407_0422	Complementation
Bt 407 $\Delta$ 1060	AH1570	None	Bt407_1060	$\Delta$ 1060
Bt 407 $\Delta$ 1060 + pXyl	AH1690	pHT304-pXyl	Bt407_1060	Control
Bt 407 $\Delta$ 1060 + 1060	AH1689	Bt407_1060- pHT304-pXyl	Bt407_1060	Complementation

**Table 5.8: Schematic overview over Bt 407 variant strains for use in the biofilm screening to determine the effect of Bt407\_0422 and Bt407\_1060 on biofilm formation.**

### 5.5.2 Electrotransformation of insert- reporter vectors into Bt407

The two gene deletion mutants of Bt 407 had been prepared by Annette Fagerlund prior to the thesis. Stock strains AH1660 (Bt407\_1060 - pHT304-pXyl in *E. coli* XL-1 blue MRF, construct cloned by Annette Fagerlund) and AH1661 (Bt407\_0422-pHT304 - pXyl in *E. coli*

XL-1 blue MRF) were grown (4.1.1) and minipreps of both plasmids prepared prior to transformation (4.3.2).

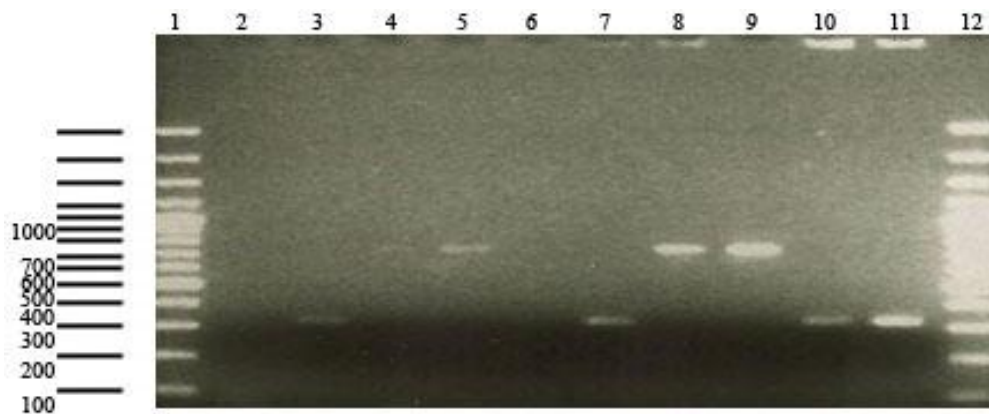
Bt 407 wildtype, Bt 407 $\Delta$ 0422 and Bt 407 $\Delta$ 1060 were prepared for electrotransformation (4.2.2). ~50 $\mu$ l cells were mixed with vector, transformed (4.11.2) and plated on LB plates with 10 $\mu$ g/ml erythromycin. The plates were grown over night at 30°C. Two to three colonies were picked and streaked onto new LB plates containing 10 $\mu$ g/ml erythromycin and grown for another 24 hours at 30°C. One to ten colonies were then inoculated in 50 $\mu$ l LB medium, 5 $\mu$ l of the inoculate was used to inoculate 5ml LB to prepare glycerol stocks, while 45 $\mu$ l was heated at 94°C for 15 minutes and 1 $\mu$ l was then used as template for colony PCR. Primers were chosen to give PCR products <1000bp.

Cells transformed with pHT304-pXyl were screened using primer set pHT304-pXyl\_forward/ S2\_pUC19R. The expected size of the PCR product was 327bp.

Cells transformed with Bt407\_0422 - pHT304-pXyl were screened using primer set pHT304-pXyl\_forward/ 00330-Bt407\_0422-R2. The expected size of the PCR product was 800bp.

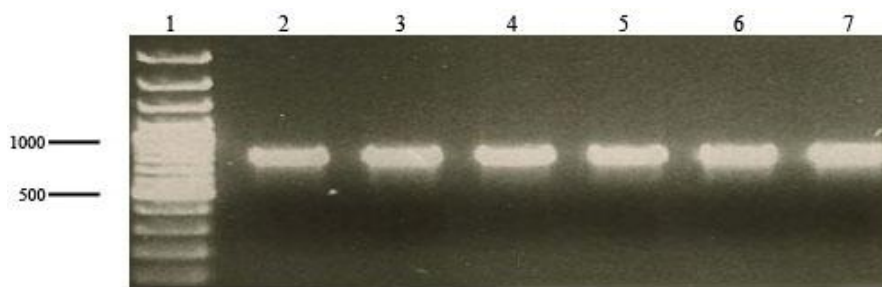
Cells transformed with Bt407\_1060-pHT304-pXyl were screened using primer set S2\_pUC19R/02561\_F2. The expected size of the PCR product was 800bp.

Altogether, the five transformations (designated AH1684, AH1686, AH1688, AH1689 and AH1690) were performed in the first round of experiments, and all five were transformed successfully (figure 31).



**Figure 31: Colony PCR of colonies of *B. thuringiensis* 407 strains with pHT304-pXyl and Bt407\_1060-pHT304-pXyl. Lanes: 1: 100bp st. (Fermentas), 2-3: AH1423 (wildtype) +pHT304-pXyl, 4-5: AH1423 (wildtype) +Bt407\_1060-pHT304-pXyl, 6-7: AH1539 (Bt 407 $\Delta$ 0422) + pHT304-pXyl, 8-9: AH1570 (Bt 407 $\Delta$ 1060) + Bt407\_1060-pHT304-pXyl, 10-11: AH1570 ( $\Delta$ 1060) + pHT304-pXyl, 12: 100bp st. (Fermentas).**

The two remaining Bt 407 variant strains, Bt 407 wildtype + Bt407\_0422 - pHT304-pXyl and Bt 407  $\Delta$ 0422 + Bt407\_0422 - pHT304-pXyl, were subsequently successfully transformed afterwards (designated AH1685 and AH1687), similar to the first Bt 407 variant strains (figure 32). Three glycerol stocks were prepared of each of the seven clones, and stored at -80°C.



**Figure 32: Colony PCR of colonies of *B. thuringiensis* 407 strains with Bt407\_0422 - pHT304-pXyl. Lanes: 1: 100bp st. (Fermentas), 2-4: AH1423 (Bt 407 wildtype) + Bt407\_0422 - pHT304-pXyl, 5-7: AH1539 (Bt 407 $\Delta$ 0422) + Bt407\_0422 - pHT304-pXyl.**



### 5.5.3 Optimization of xylose concentration for overexpression and complementation studies

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To be able to do overexpression and complementation studies with Bt 407- based strains containing the Bt407\_4022 and Bt407\_1060 - pHT304-pXyl, it was necessary to do a xylose titration study to ascertain the xylose concentration needed for appropriate expression levels.

The experimental setup of the microtiter plate screening assay is shown in table 5.9. We started out doing a complementation study of Bt 407 $\Delta$ 1060 with Bt407\_1060 - pHT304-pXyl (AH1689) set up according to the protocol (4.13). To be certain that the pHT304-pXyl vector itself did not change biofilm formation, Bt 407 $\Delta$ 1060 with pHT304-pXyl (AH1686) was included for control. Four identical microtiter plates were set up, and biofilm formation was measured after 24, 48, 72 and 96 hours, respectively (4.13).

However, the biofilm formation at the air-liquid interface for this assay was highly unstable. Visual inspection of the plates, after initial wash and staining with crystal violet, showed that large fragments of biofilm ring had been lost during plate handling. The results from OD measurements of the Bt 407 wt, Bt 407 $\Delta$ 0422 and Bt 407  $\Delta$ 1060 strains did not comply with results generated in previous assays in the lab (5.2). The unstable biofilms were observed in every well, both the strains where xylose was added and the strains without xylose.

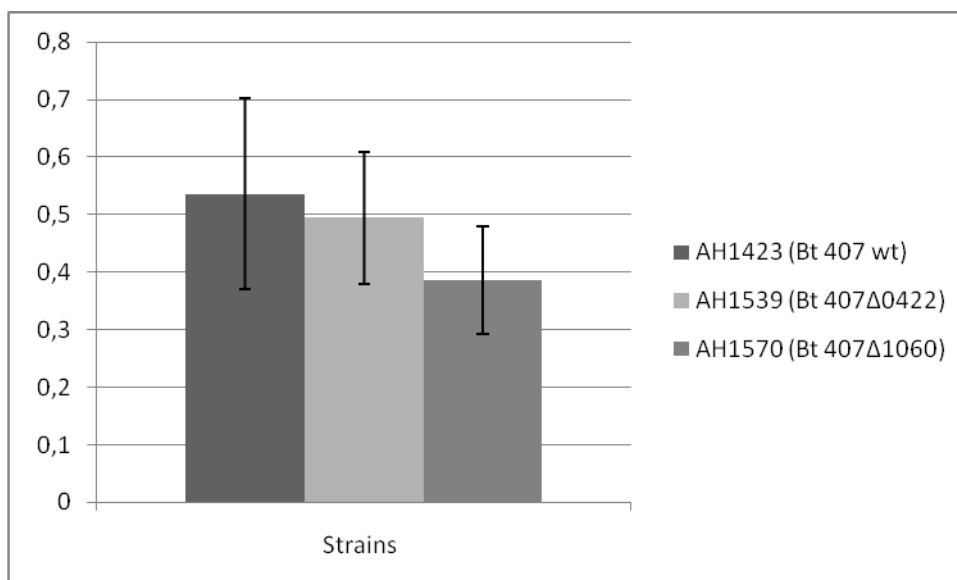
	1	2	3	4	5	6	7	8	9	10	11	12
A	Bactopeptone (blank)	Bt 407 wt (AH1423)	Bt 407Δ0422 (AH1539)	Bt 407Δ1060 (AH1570)	AH1690 (0mM xylose)	AH1690 (0,03mM xylose)	AH1690 (0,1mM xylose)	AH1690 (0,3mM xylose)	AH1690 (1mM xylose)	AH1690 (3mM xylose)	AH1690 (10mM xylose)	10μM xylose in bactopeptone
B												
C												
D												
E												
F												
G												
H												

**Table 5.9: Microtiter plate design for complementation assay for deletion of Bt407\_1060 with Bt 407 wildtype, Bt 407Δ0422 and Bt 407Δ1060 strains. AH1689: Bt407Δ1060 with Bt407\_1060-pHT304-pXyl, AH1690: Bt407Δ1060 with pHT304-pXyl. Wells containing bacteria carrying plasmids were added 10μg/ml erythromycin.**

A repeated biofilm assay designed in the same manner as the first xylose titration assay (5.5.3, table 5.9) showed the same problems with biofilm stability, as did a similar complementation titration assay with the Bt407Δ0422 strains containing the Bt407\_0422-pHT304 - pXyl plasmid.

### 5.5.4 Control of biofilm assay method

To control whether another external unknown factor was affecting biofilm formation, a microtiter plate assay was set up in the same manner as the previous biofilm screening assay with Bt 407 wildtype (AH1423) and Bt 407 $\Delta$ 0422 (AH1539)/ Bt 407 $\Delta$ 1060 (AH1570) gene deletion mutant strains. Visual inspection and OD measurements showed no problem with biofilm stability after 24 hours of growth at 30°C (figure 33). This small study did not show the significant difference between Bt 407 wt and Bt 407 $\Delta$ 1060 on biofilm formation as was observed in the biofilm screening assay described in 5.2, but the aim here was to ascertain whether the biofilm formation was disrupted in the same manner as the xylose titration assays or not. No such disruption was observed.



**Figure 33: Control of biofilm screening assay results using Bt 407 wt (AH1423), Bt 407 $\Delta$ 0422 (AH1539) and Bt 407 $\Delta$ 1060 (AH1570). The bacteria were applied to a microtiter plate (8 wells each) for 24 hours of growth at 30°C, washed, stained and OD measured as stated in the protocol (4.13).**

Since no problems were present in the biofilm assay described above, it was speculated that xylose or erythromycin could have a destabilizing effect on biofilm formation since only the biofilm assays where these two chemical components were present exhibited unstable biofilm formation. A second theory was that the design of the plates itself, with wells containing bacteria adjacent to each other could result in biofilm instability. The stable biofilm screening assays had wells containing bactopectone medium to separate the wells containing bacteria. The third theory was that the bactopectone medium used was not prepared correctly. A 24h and 48h control of these three theories did not resolve the biofilm instability issue (not shown).

## **5.6 Cloning of promoter regions for Bt407\_0422 and Bt407\_1060 into pHT304-18z reporter vector to create lacZ fusion constructs**

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In order to study the regulation of Bt407\_0422 and Bt407\_1060 in *Bacillus thuringiensis* 407, it was decided that the promoter regions identified upstream of these two genes that was similar to the Bc1 and Bc2 riboswitches described by Sudarsan [63] should be cloned in a TOPO II vector and transferred, after proper verification of correct sequence, to the reporter vector pHT304-18z in order to do lacZ studies.

pHT304-18z is a shuttle vector constructed for cloning in *B. thuringiensis* and *E. coli*. It contains two antibiotic resistance markers, an ampicillin resistance gene functional in *E. coli* and an erythromycin resistance gene functional in both *E. coli* and *B. thuringiensis*. It also contains a promoterless *lacZ* gene which codes for a  $\beta$ -galactosidase enzyme [71].

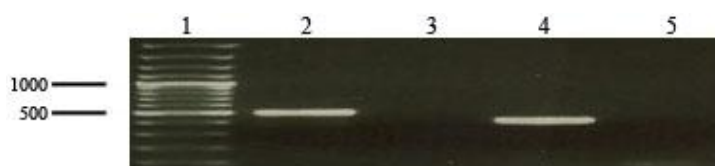
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### 5.6.1 PCR

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Promoter regions, hereby designated as promBt407\_0422 for the promoter region upstream from Bt407\_0422 and promBt407\_1060 for the promoter region upstream from Bt407\_1060, were amplified by PCR (4.5) with primers P-Bt407\_0422-F/P-Bt407\_0422-R for promBt407\_0422 and P-Bt407\_1060-F/P-Bt407\_0422-R for promBt407\_1060 (3.1.3), using genome DNA from *B. thuringiensis* 407 as template. The estimated lengths of the PCR products were expected to be 498bp and 404bp, respectively.

10µl of the PCR products were applied on an agarose gel for electrophoresis, along with a negative PCR control lacking template DNA (figure 34) for each primer set.



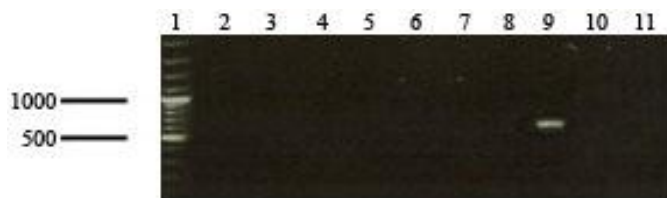
**Figure 34: Initial PCR of promBt407\_0422 and promBt407\_1060. Lanes: 1: 100bp st. (Fermentas), 2: PCR product of promBt407\_0422, 3: Negative control of PCR of promBt407\_0422 without genome as template, 4: PCR product of promBt407\_1060, 5: Negative control of PCR of promBt407\_1060 without genome as template.**

### 5.6.2 TOPO cloning

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The PCR products covering the promoter regions (5.6.1) were then inserted into a TOPO II vector (4.12), hereby designated promBt407\_0422 – TOPO and promBt407\_1060 – TOPO,

respectively, and the constructs were transformed into electrocompetent *E. coli* XL-1 blue MRF- cells (4.11.1) and plated on LB plates containing 100µg/ml ampicillin / 50µg/ml kanamycin. Several colonies were picked and screened by colony PCR (4.5) with primers S2\_pUC19R/P-Bt407\_0422-R for promBt407\_0422 and S2\_pUC19R/P-Bt407\_1060-R for promBt407\_1060 (3.1.3). The PCR products were checked on agarose gels for size and specificity. The expected sizes of the PCR products were 643bp and 549bp, respectively. The colony PCR revealed one positive colony for each promoter region (figure 35 and 36).



**Figure 35: Colony PCR of colonies supposed to contain promBt407\_0422 – TOPO.**  
Lanes: 1: 100bp st. (Fermentas), 2-11: Colony 1-10.

asd



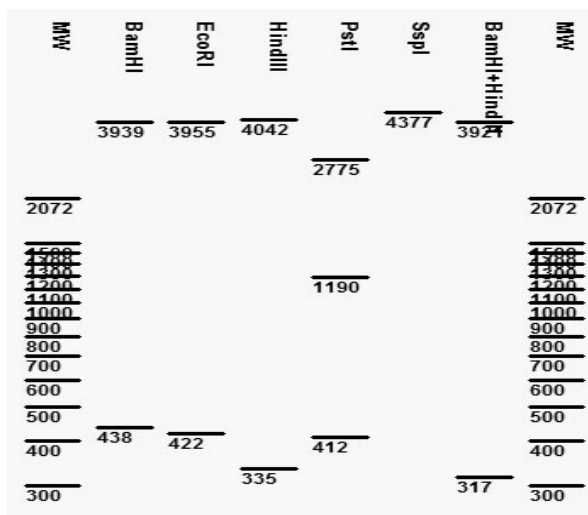
**Figure 36: Colony PCR of colonies supposed to contain promBt407\_1060 – TOPO.**  
Lanes: 1: 100bp st. (Fermentas), 2-11: Colony 1-10.

### 5.6.3 Restriction test

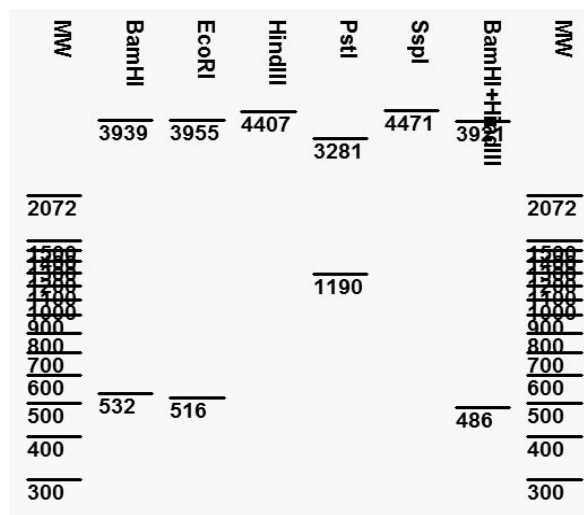
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In order to verify whether the promoter regions had been inserted correctly into the TOPO vector, restriction digest tests were done using enzymes known to be present in the

promBt407\_0422 – TOPO and promBt407\_1060 plasmids. Plasmid minipreps of each promoter- TOPO construct were prepared (4.3.2).

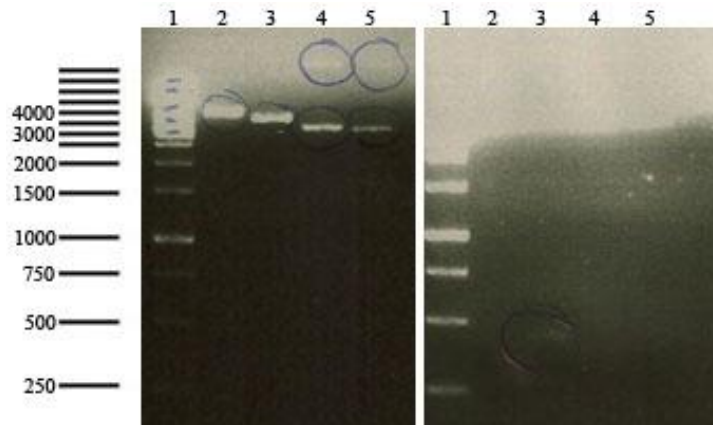


**Figure 37: *In silico* restriction digest test of the promBt407\_1060 – TOPO plasmid.**



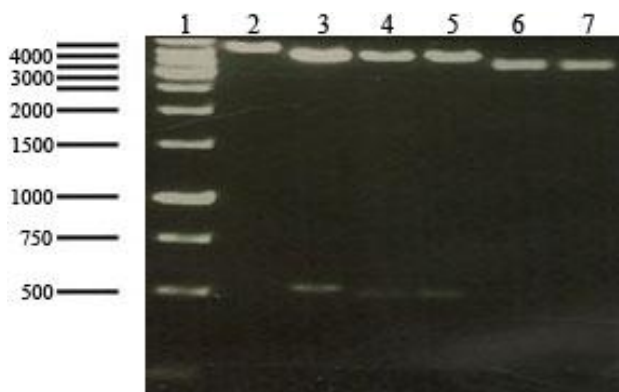
**Figure 38: *In silico* restriction test of the promBt407\_0422 – TOPO plasmid.**

A restriction test (4.7) with a 1:10 dilution of the minipreps, using *Bam*HI and *Eco*RI for each promoter, gave bands with correct sizes for promBt407\_1060 - TOPO, but not for promBt407\_0422 – TOPO (figure 37,38 and 39).



**Figure 39: Restriction test of minipreps from 2 colonies supposed to contain promBt407\_0422 – TOPO and promBt407\_1060 – TOPO. Lanes. 1: 1kb st. (Fermentas), 2: promBt407\_0422 cut with *Bam*HI, 3: promBt407\_1060 cut with *Bam*HI, 4: promBt407\_0422 cut with *Eco*RI, 5: promBt407\_1060 cut with *Eco*RI. Both photos show the same gel, but more light is applied to the photo on the right to illuminate the 438bp band produced when promBt407\_1060 - TOPO is digested with *Bam*HI.**

An additional restriction test using plasmid DNA from two additional colonies supposed to contain promBt407\_0422 – TOPO, using the enzymes *Bam*HI and *Eco*RI, respectively, resulted in the identification of one positive clone (figure 40).



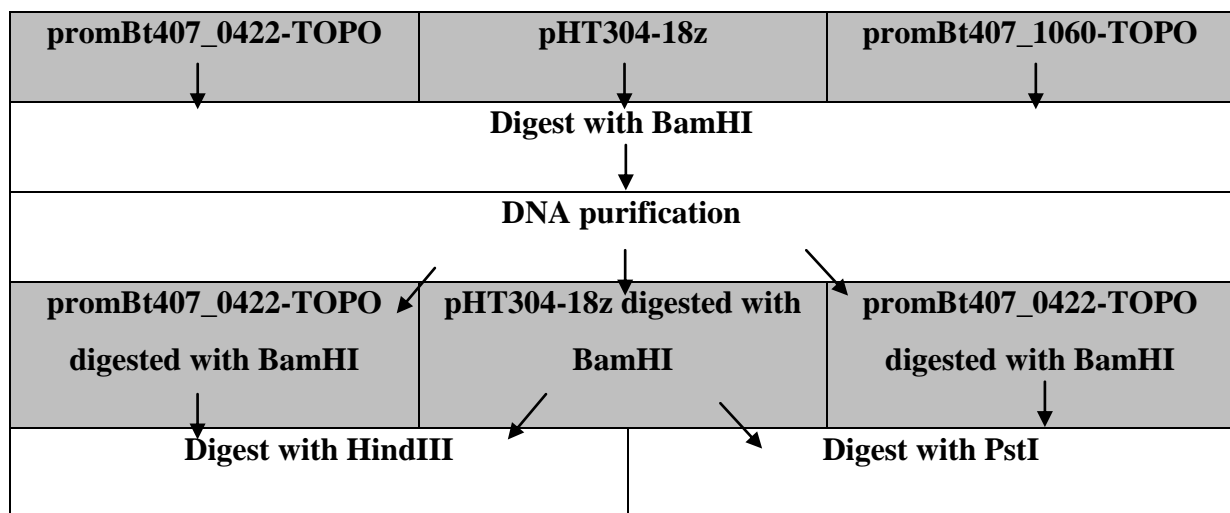
**Figure 40: Restriction test of minipreps made from 2 colonies supposed to contain promBt407\_0422 – TOPO. Lanes: 1: 1kb st. (Fermentas), 2: Colony 1 cut with *Bam*HI, 3: Colony 2 cut with *Bam*HI, 4: Colony 1 cut with *Eco*RI, 5: Colony 2 cut with *Eco*RI, 6: Uncut miniprep from colony 1, 7: Uncut miniprep from colony 2.**



An additional restriction test with *Pst*I and *Ssp*I for the previously identified, positive colonies for each promoter gave a positive result (not shown). It was decided to continue to transfer the promoter to the reporter vector, pHT304-18z.

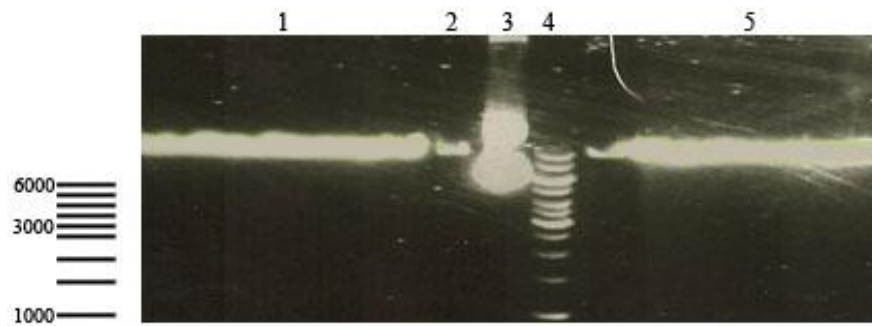
#### 5.6.4 Large digest, cutting, purification and ligation of insert and reporter plasmids

The aim of the next step was to digest promBt407\_0422 - TOPO and pHT304-18z with *Bam*HI and *Hind*III, purify and finally ligate the DNA fragments together. Similarly, promBt407\_1060-TOPO and pHT304-18z would be digested with *Bam*HI and *Pst*I and be purified and ligated (table 5.10).



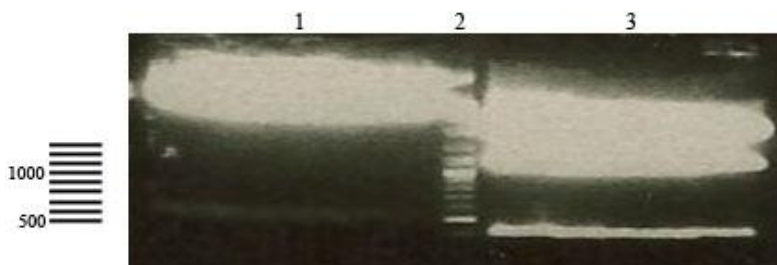
**Tabell 5.10: Schematic overview of transfer of inserts to reporter vector**

Both inserts (~25µg) and reporter vector (~2µg) was digested with *Bam*HI. All reactions were purified using a commercial DNA purification kit (3.7). The reporter vector sample was digested with *Bam*HI and equally split into two different reactions; one reaction was digested with *Hind*III and one reaction with *Pst*I (table 5.10). The double digested vector samples were then separated on a 0.7% SeaKem GTG preparative agarose gel (4.6)(figure 41).



**Figure 41: Large restriction digests. Lanes: 1: pHT304-18z digested with *Bam*HI and *Hind*III, 2: uncut vector, 3: 1kb st. (Fermentas), 4: pHT304-18z digested with *Bam*HI and *Pst*I**

*Bam*HI- digested promBt407\_0422 - TOPO and promBt407\_1060 - TOPO were then digested with *Hind*III and *Pst*I, respectively, and separated on a 1.2% NuSieve GTG preparative agarose gel (4.6)(figure 42).



**Figure 42: Large digest of promBt407\_0422 – TOPO and promBt407\_1060 – TOPO. Lanes: 1: promBt407\_0422 –TOPO digested with *Bam*HI and *Hind*III, 2: 100bp st. (Fermentas), 3: promBt407\_1060 – TOPO digested with *Bam*HI and *Pst*I.**

DNA bands were excised from the gels and purified with a commercial DNA purification kit (4.3.1). After purification of DNA from the gel, the different DNA fragments were quantified by spectrophotometry or quantitative gel electrophoresis (4.4.1 and 4.4.2), Two ligation reactions were set up, one with 1:3 molar ratio of vector/insert and one with 1:5 molar ratio of vector/insert. The reactions were then ligated at 16°C overnight (4.8). The ligation

reactions were then dialyzed on filter paper for 30 minutes the next day, transformed into *E. coli* (4.11.1) and plated on LB plates containing 100µg/ml ampicillin / 200µg/ml erythromycin / 80µg/ml X-gal. After incubation at 37°C overnight, no colonies were detected.

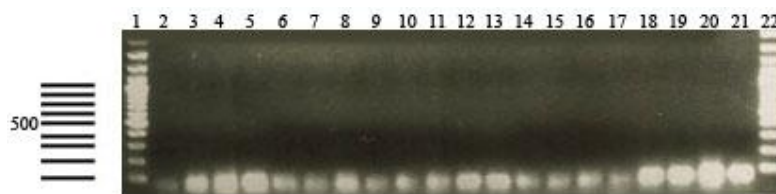
An additional restriction test revealed that promBt407\_0422-TOPO and promBt407\_1060-TOPO had both restriction digest sites intact (not shown). However, sequence analysis revealed a single mutation in the promoter region of promBt407\_1060-TOPO (appendix 1 and 2). An additional analysis of the original PCR template turned out to contain no mutations (appendix 5). It was decided to start the cloning procedure for promBt407\_1060 from the beginning, following the same protocol. A miniprep of promBt407\_1060 - TOPO was generated from a new colony, and restriction tested with *Bam*HI, *Pst*I and *Ssp*I (not shown). It was decided to again attempt to transfer the promoter regions to the pHT304-pXyl reporter vector.

Two addition attempts turned out negative, visual inspection of the LB plates showed a viscous layer of cell debris on the plates. The third attempt included several ligation controls where the vector pHT304-18z was digested with *Bam*HI and ligated together, as well as double digested promBt407\_0422 – TOPO and promBt407\_1060 was transformed without ligase added to the ligation reaction. The controls suggested that nothing was wrong with the procedure. It was then discovered by comparing protocols in the lab that the erythromycin concentration for growth of *E. coli* containing pHT304-18z constructs preferably should've been 400µg/ml, not 200µg/ml.

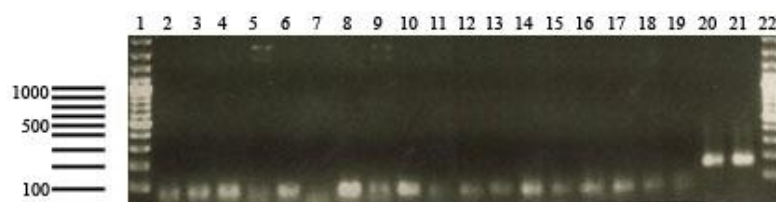
For the last attempt, the amount of erythromycin was changed from 200 to 400µg/ml erythromycin. Transformed cells plated on LB plates containing 100µg/ml ampicillin / 400µg/ml erythromycin / 80µg/ml X- gal gave several colonies (>100) for both promBt407\_0422 - pHT304-18z and promBt407\_1060 - pHT304- 18z constructs.

### 5.6.5 Colony PCR screening of pHT304-18z constructs

Twenty colonies of each construct were picked and screened by colony PCR using primers PU and OVG (3.1.3). The PU primer is located upstream from the insert site in pHT304-18z while OVG is located downstream. The expected size of the PCR products was between 500-1000bp. Electrophoresis (figure 43 and 44) indicated that none of the colonies contained the expected constructs.



**Figure 43: Colony PCR of colonies supposed to contain promBt407\_0422 - pHT304-18z. Lanes: 1: 100bp st. (Fermentas), 2-21: Colony 1-20, 22: 100bp st. (Fermentas).**



**Figure 44: Colony PCR of colonies supposed to contain promBt407\_1060 - pHT304-18z. Lanes: 1: 100bp st. (Fermentas), 2-19: Colony 1-18, 20-21: Positive control with midiprep of empty vector (pHT304-18z), 22: 100 bp st. (Fermentas).**

The colonies were screened by two additional primer sets each; both sets contained one primer upstream and one primer downstream of the insert site in pHT304-18z, but none of the PCR's produced the expected bands, indicating that the clones did not carry the expecting

plasmids. Plasmid minipreps were prepared (4.3.2) followed by restriction testing using *Bam*HI, *Hind*III and *Pst*I, but no bands were detected after gel electrophoresis (4.6).

Due to lack of time, no further work was done on the promBt407\_0422 and promBt407\_1060 inserts.

## Chapter 6: Discussion

### 6.1 Role of Bt407\_0422 and Bt407\_1060 in biofilm formation by *B. thuringiensis* 407

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Preliminary results by Annette Fagerlund indicated that gene deletion of Bt407\_1060 in *B. thuringiensis* 407 resulted in lower biofilm formation after 24, 48 and 72 hours of growth compared to wt. Gene deletion of Bt407\_0422 seemed to result in a slightly lower biofilm formation after 24 hours compared to wt, but not after 48 and 72 hours.

#### 6.1.1 The role of Bt407\_1060 in biofilm formation by *B. thuringiensis* 407

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The more extensive screening performed for this thesis (5.2.2) confirmed that gene deletion of Bt407\_1060 in *B. thuringiensis* 407 results in significantly lower biofilm formation after 24, 48 and 72 hours using a microtiter plate screening assay *in vitro* (4.13). However, the mutant was able to produce biofilm to a certain extent. Bt407\_1060 should, therefore, be considered an important/highly influential gene in biofilm formation by *B. thuringiensis* 407, but not essential.

BLAST search of the protein sequence (5.1.1) revealed that orthologs of Bt407\_1060 are not heavily distributed throughout the nineteen strains with closed genomes included in the search, and the presence of these orthologs did not absolutely correlate with strong biofilm producer capabilities (table 6.1). A biofilm producing strain such as *B. cereus* ATCC 10987 did not contain any Bt407\_1060 ortholog, which means that Bt407\_1060 is not a determining factor of biofilm formation in this strain.

Strains	Biofilm	Bt407_1060 ortholog
<i>Bacillus cereus</i> ATCC 10987	<b>Positive (+) [19, 90]</b>	Not present
<i>Bacillus cereus</i> ATCC 14579	Negative (-) [90]	<b>Present</b>
<i>Bacillus cereus</i> AH187	Negative (-) [90]	Not present
<i>Bacillus cereus</i> NVH391-98	Negative (-) [90]	Not present
<i>Bacillus thuringiensis</i> 407	<b>Positive (+) [90]</b>	<b>Present</b>
<i>Bacillus anthracis</i> Sterne	Positive (+) [92]	Not present

**Table 6.1: Overview of a selection of *B. cereus* group strains where data on 1) biofilm formation and 2) the presence of Bt407\_1060 orthologs is available.**

In order to explain the results from the functional studies, information regarding the putative properties of Bt407\_1060 and knowledge of key factors in biofilm formation by *B. thuringiensis* 407 is important. Bt407\_1060 and its orthologs in other sequenced strains were, initially, annotated as a collagen-binding protein. Analysis of the Bt407\_1060 protein sequence (5.1.1) showed that it contains two domains which closely resemble the collagen-binding domain and Cna-like B-type domain characterized in *Staphylococcus aureus* [83], constituting the ligand binding domain and the domain responsible for elevation of the binding domain away from the cell wall, respectively. Furthermore, an LPXTG- motif was identified near the C- terminal which meant that it may be anchored extracellularly to the cell wall. Supporting this theory, a SignalP cleavage site was also discovered which meant that the protein may be transported by the general bacterial secretion apparatus (Sec). Based on these results, it seems probable that the initial annotation is correct and the Bt407\_1060 protein may be involved in adhesion/cell- to- cell interactions, however, whether is the real ligand remains to be functionally established.

The importance of adhesion for establishing a monolayer attached to a surface and cell- to- cell interactions in development of multilayer biofilms is well known [32]. Not much

research has been done on protein- mediated adhesion by *B. cereus* and *B. subtilis*, biofilm research in *Bacillus* species have focused almost exclusively on components of the biofilm matrix and their regulators. However, it has been shown that *B. subtilis* SpoOA gene deletion mutants were not able to develop biofilm beyond a monolayer [93], and it was suggested that this effect was due to impaired cell-cell interaction. EPS and TasA, two components regulated by SpoOA and is secreted out of the bacterium , have been shown to be important since mutant strains are not able to produce a stable biofilm [40].

In other gram- positive bacteria, such as *S. epidermidis* and *S. aureus*, initiation of biofilm formation has been linked to cell-surface interactions [94]. Several studies have implicated a polysaccharide intercellular adhesin (PIA) to be important for cell- cell- interactions and formation of cell aggregates on a surface [94, 95]. Deletion of the *ica* locus coding for this adhesin resulted in impaired biofilm formation compared to wildtype in one study [96]. Additionally, the protein Bap (biofilm associated protein) is a surface protein with adhesin properties that has been observed to affect biofilm formation on a polystyrene surface by *S. aureus* [62].

To conclude, results from screening assays indicate that Bt407\_1060 may have a functional role in biofilm formation since a *B. thuringiensis* 407  $\Delta$ Bt407\_1060 mutant exhibited impaired biofilm formation. The theoretical adhesion properties of the Bt407\_1060 protein, as well as the potential role of c-di-GMP in regulation of the expression of Bt407\_1060, are intriguing since research concerning adhesion in biofilm formation by *Bacillus* species is scarce. However, complementation studies are necessary to rule out any possible polar effects from unexpected, additional mutations arising from the gene deletion procedure.

### 6.1.2 The role of Bt407\_0422 in biofilm formation by *B. thuringiensis* 407

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The more extensive screening in this thesis (5.2.2) showed no difference in biofilm formation between Bt 407 wt and the  $\Delta$ Bt407\_0422 gene deletion mutant.



This is in conflict with previous preliminary assays where a small difference was observed during the first 24 hours of growth. Thus, it seems probable that Bt407\_0422 is not an essential gene in biofilm formation by *B. thuringiensis* 407. However, the results do not rule out that an effect may be observed when applying other screening techniques.

Annotation in sequenced *B. cereus* group genomes indicated that the Bt407\_0422 protein was a methyl-accepting chemotaxis protein (MCP). Analysis of the Bt407\_0422 protein sequence (5.1.2) revealed a HAMP domain and other intracellular domains usually found in chemotaxis proteins. Furthermore, a signal cleavage site and two transmembrane regions were identified, indicating that the protein is transported by the general secretion apparatus (Sec) out of the cell and constitute a transmembrane protein, respectively. The positioning of these domains indicated that Bt407\_0422 contains an extracellular helix with sensory functions and an intracellular region with signalling properties. This correlates well with the general model for methyl- accepting chemotaxis proteins [4] and the initial annotation is considered correct.

Chemotaxis is responsible for sensing chemical gradients in close proximity to the cell and, through intracellular signaling for motility towards favorable conditions by affecting the flagellar apparatus [97]. Bacteria usually contain several different receptors; 10 different chemotaxis receptors have been identified in *B. subtilis* which are able to sense a wide range of different ligands [97]. Methyl-accepting chemotaxis proteins undergo a conformational change after binding of ligand, and, through several signalling steps, result in phosphorylation of the effector protein CheY which determines the direction of flagellar rotation [4].

A role of chemotaxis in biofilm formation has not been described. One possibility could be that chemotaxis is required for the bacteria to swim towards nutrients associated with a surface. Chemotaxis is linked to, and influences motility by regulation of flagellar movement, but one study done in *E. coli* showed that motility and flagellar movement mutants produced a severely impaired biofilm while chemotaxis mutants produced biofilms similar to wt [98].

Transcriptional studies have indicated that genes encoding methyl- accepting chemotaxis proteins are highly activated during monolayer formation [31]. This suggests that chemotaxis may have a role either in further development into a multilayer biofilm or in dispersion of the biofilm, two actions which require motility.

*B. thuringiensis* 407 is motile, contains an extensive flagellar apparatus and shows swimming/swarming behaviour in vitro [99]. *B. subtilis* cells with a dysfunctional flagellar apparatus exhibit lower biofilm forming capabilities compared to wt [100]. In that study, chemotaxis is shown not to be a determining factor for biofilm formation. Thus, the results in *B. subtilis* correlate well with the results experienced with the  $\Delta$ Bt407\_0422 mutant strain. The design of the microtiter plate screening assay for this thesis, however, is arguably not the ideal model to investigate whether the theoretical chemotaxis protein Bt407\_0422 has an effect on biofilm formation. Since one of the key factors for initiation of biofilm formation by motile bacteria is the ability to move towards a surface or the air-liquid interface, the rotation during incubation could theoretically bring most cells in close proximity by rotational force.

To conclude, Bt407\_0422 is a gene which is most likely coding for a methyl- accepting chemotaxis protein. However, functional studies did not show any effect on biofilm formation. This was not entirely unexpected since several studies on biofilm formation have concluded that chemotaxis was not essential for biofilm formation. Since it is possible to observe differences in biofilm formation behaviour in different screening systems [32], it would be interesting to perform additional studies in other systems where chemotaxis may be more favourably modelled.

### 6.1.3 The role of c-di-GMP on Bt407\_1060 and Bt407\_0422 in biofilm regulation

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In gram-negative bacteria, the general effect of c-di-GMP on biofilm formation is that elevated c-di-GMP levels in the cell increase adhesion, while suppressing motility [66], causing a shift from a motile lifestyle to a biofilm-forming lifestyle. However, this seemingly doesn't apply to the control of Bt407\_0422 and Bt407\_1060, where the opposite effect was seemingly found. Elevated c-di-GMP was found, by Sudarsan and co-workers, to increase Bc1 (c-di-GMP- responsive "on" switch) activity [63] which will increase Bt407\_0422 expression and, therefore, potentially increase chemotaxis. Elevated c-di-GMP was found to decrease Bc2 (c-di-GMP- responsive "off" switch) activity [63] which will suppress Bt407\_1060 expression and, therefore, potentially reduce adhesion [63]. Lower c-di-GMP will produce the opposite effects. C-di-GMP levels and regulatory processes in different environments in *Bacillus* species are, however, poorly investigated. It has been claimed that extracellular c-di-GMP added to the growth medium inhibits biofilm formation by *Staphylococcus aureus* [101]. This study is, however, more concerned about the action of c-di-GMP on cell-to-cell interaction and biofilm formation as a possible drug candidate, and the intracellular signalling during biofilm formation has not been properly investigated. In the paper, the researchers pondered whether the observed effect was due to extracellular rather than the more biologically relevant intracellular actions of c-di-GMP [101].

In order to enhance our understanding of Bt407\_0422 and Bt407\_1060, further studies on c-di-GMP level variations in *Bacillus* species would be of great interest and is one of the future aspects of this project (6.4).

## **6.2 Cloning procedure of Bt407\_1060 and Bt407\_0422 promoter regions in pHT304-18z vectors**

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The original aim, to produce constructs of the promoter regions of Bt407\_0422 and Bt407\_1060 with the pHT304-18z reporter vector, was not met. There are several reasons for this. The first, and most important reason, is the complexity of the cloning procedure itself which meant that initial negative results was more likely to be a result of imprecise laboratory technique. Although most procedures in the beginning included negative and/or positive controls if it was deemed prudent, troubleshooting was not always done after initial negative attempts.

The initial TOPO cloning procedure did not present any problems. The qualitative control of promoter-TOPO constructs, however, turned out to be insufficient. The procedure was based on restriction testing with a selection of enzymes, including the restriction sites incorporated in the primers for the initial PCR. Restriction testing is a fast and cheap technique that may reveal if the bulk of the DNA is correct, and if the expected restriction sites are present. However, point mutations in the sequence might be left undiscovered if DNA sequencing is not applied, as it did with the sequence for the promoter region for Bt407\_1060. When obtained, it was discovered that two point mutations were present and the entire cloning procedure up to that point had to be redone.

The cloning procedures could then progress to the transfer of promoter regions from the TOPO vector to the pHT304-18z vector. Several attempts were made following the original protocol but no colonies were detected. This procedure was done twice prior to troubleshooting. Control of the ligation procedure with positive controls (single- cut vector which was ligated and produced colonies) and negative controls (double- cut vectors without insert and which produced no colonies) suggested no systematic problems with the procedure. Comparison of other lab protocols revealed that 400µg/ml erythromycin, not 200µg/ml as was stated in the original protocol, was prudent. A layer of cell debris had been

observed on the LB plates during previous attempts, the same phenomenon was observed with LB plates older than a month containing 400µg/ml erythromycin. One suggestion is that the previous attempts possibly were successful in joining the promoter regions with the reporter vector, but the lack of antibiotics might have resulted in low natural selection pressure which meant that bacteria not containing the plasmids were not eradicated as thorough as they should have been. However, the cloning attempts with 400µg/ml erythromycin turned out to only give contaminants, and extensive testing with colony PCR, using primers designed to detect the other vectors employed regularly in the lab, and restriction digests gave no answers to what these colonies contained. In other studies, such as the initial construction of the shuttle vector pHT304 [71], 150µg/ml erythromycin was used.

Due to time issues, focus was shifted from the cloning procedure to get the Bt407\_0422 and Bt407\_1060 pHT304-pXyl constructs ready for biofilm assay.

## 6.3 Complementation/overexpression of Bt407\_0422 and Bt407\_1060 proteins in *Bacillus thuringiensis* 407 (Bt 407)

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In order to control whether the effect observed by the  $\Delta$ Bt407\_1060 and  $\Delta$ Bt407\_0422 gene deletion mutants was due to some other, external factor present, both genes were inserted into pHT304-pXyl expression vectors in order to do complementation studies in the mutant strains and overexpression in the Bt 407 wildtype strain. The cloning procedure was successful, but the necessary titration of xylose with the constructs was not.

### 6.3.1. Cloning of Bt407\_0422 into pHT304-pXyl shuttle vector

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The cloning of Bt407\_0422 into pHT304-pXyl was riddled with similar problems as the promoter regions (6.2), however, there were some additional issues which had to be solved. At least two attempts of transferring the Bt407\_0422 fragment to TOPO to pHT304-pXyl were performed before it was discovered that the restriction site for *Xba*I was missing from the TOPO construct. The reason for this may have been either an error in the primer itself, or a point mutation that developed later in the procedure. The cloning procedure up to this point had to be redone.

Although the attempts to transfer the Bt407\_0422 insert into pHT304-pXyl were riddled with difficulties, one cloning attempt done by Annette Fagerlund was successful. Sequence analysis of these clones revealed two point mutations in the sequence. The most probable

reason for this was the use of Dynazyme II polymerase for the initial PCR reaction (Finnzymes, [102], an enzyme with less proofreading properties compared to e.g. *Pfu* (Promega, [103]) which contains 3'-5' exonuclease activity. In the end, the mutations were dealt with using mutagenesis; the construct was finished and transformed into *E. coli* and *B. thuringiensis* 407.

### 6.3.2 Xylose titration of Bt407\_0422 and Bt407\_1060- pHT304- pXyl constructs

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Xylose titration experiments, where the goal was to ascertain optimal concentrations of xylose in the bactopectone medium for expression of Bt407\_0422 and Bt407\_1060 from the pHT304-pXyl shuttle vector, for complementation and overexpression studies, respectively, did not work. The reason for this is enigmatic. The biofilm assay performed satisfactory when comparing the  $\Delta$ Bt407\_1060 and  $\Delta$ Bt407\_0422 gene deletion mutant strains to wt, but even these strains, with no xylose or antibiotics added to the wells, produced unstable biofilms when xylose or antibiotics were added to other strains in other wells on the same microtiter plate. This particular effect was observed no less than three times, followed by a control screening with Bt 407 wt and the gene deletion mutants Bt 407  $\Delta$ Bt407\_1060 and  $\Delta$ Bt407\_0422, where no xylose or antibiotics was added to the plate, which reproduced the formation of stable biofilms seen in the first screening assay experiment (5.2.2).

It was speculated whether xylose, or the presence of antibiotics for selection of bacteria containing plasmids, could have an inhibitory effect on biofilm formation. However, it is known that most *Bacillus* species utilize glucose, not xylose, as a carbon source [17]. Glucose is known to inhibit biofilm formation in *Bacillus subtilis* [53], but this mechanism does not seem to be affected by xylose, and there are no other data concerning this available. Additional experiments are absolutely required to resolve whether the effect of xylose, or antibiotics for plasmid selection, is truly inhibitory of biofilm formation for *B. thuringiensis*.

## 6.4 Future aspects

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In the end, some of the aims of the thesis were met and some were not. There are several interesting possibilities to expand on the work done in this thesis. The first priority would be to ascertain the correct xylose concentration for the Bt407\_0422 and Bt407\_1060 – pHT304-pXyl vectors and proceed with the complementation and overexpression studies. Due to the collected results from the functional studies, complementation of Bt407\_1060 would be of great interest in order to ratify the results from the functional studies (5.2.2).

The next priority for future work on the biofilm project would be to finish the transfer of the promoter regions from the TOPO constructs to the pHT304-18z reporter vector. The finished constructs could be used in lacZ studies to 1) investigate time of activation during growth or 2) transform the constructs into c-di-GMP synthesis gene deletion mutants and identify what genes in *Bacillus thuringiensis* 407 regulate the riboswitches controlling Bt407\_1060 and Bt407\_0422 expression.



# Appendixes

## Appendix 1: Sequence analysis of promBt407\_0422-TOPO (9-11-2009)

Original sequence; pCR II-TOPO-promoter of Bt407\_0422 in Bt 407, forward direction:

aacaagcttcgaaattcattagttaagatatattcgaatagttaattgaatgaaaattaaataaacccgaagaaatccttggtgttatac  
 atttttattatataacacaaaaaaagtaacattcacgtacttttgaaattatttgaaaaagcaatgcattaaaaattgtatttatctctaaa  
 taagtagacattttgatttatatgttgtaatctttgaaagagaataaattaattctcatagaactcccatatcggtcaactcggtCAGCGA  
 GAAAGGCAAACCTGATGGAAACATGAGGACGCAAACTACAGGAGCTAAGGTCTG  
 AAAGGCTACGCTAGCCAGTTACCGGACGgtaggggttggtcttgccaatgcttttacattggctttttttattc  
 tcgaacatattgagaacattttcaacaaaatattcatatattttgtttttgtttatgtaattaaggaaggtggtatcctaa

Obtained sequence, pCR II-TOPO-promoter of Bt407\_0422 in Bt 407,  
 S2\_pUC19R:

aagcttcgaaattcattagttaagatatattcgaatagttaattgaatgaaaattaaataaacccgaagaaatccttggtgttatacattt  
 ttattatataacacaaaaaaagtaacattcacgtacttttgaaattatttgaaaaagcaatgcattaaaaattgtatttatctctaaataa  
 gtagacattttgatttatatgttgtaatctttgaaagagaataaattaattctcatagaactcccatatcggtcaactcggtcagcgagaaagg  
 caaactgatggaaacatgaggacgcaaaactacaggagctaaggtcgaaaggctacgctagccagttaccggacggatagggttg  
 tcttgccaatgcttttacattggctttttttattctcgaacatattgagaacattttcaacaaaatattcatatattttgtttttgtttatgta  
 attaaggaaggtggtatcctaa

Emboss analysis (<http://emboss.bioinformatics.nl/emboss/output/201692/>, 9/11-2009)  
 revealed no mutations.

Original sequence; pCR II-TOPO-promoter of Bt407\_0422 in Bt 407,  
complementary, reverse strand:

---

```
ggatccaccttccttaattaacataaacaacaaaaacaaaatatatgaatatttgtttgaaaatgtttctcaatatgttcgagaataaaaaaaaa  
gccaatgtaaaagcattggccaagaccaaccctatccgtccggaactggctagcgtagccttcgaccttagctcctgtagtttgcgctc  
ctcatgtttccatcagtttgcctttctcgctgaacgagttgaacgatatgggagttctatgagaattaatttattctcttcaaagattacaacat  
ataaatcaaaatgtctacttatttagagatataaatacaaattttaatgcattgcttttcaaaataatttcaaaaagtagctgaatgttactttt  
ttgtgttatataataaaaaatgtataaacaacaaggatttcttcgggtatttatttaatttcattcaaattaactattcgaatatacttaactaa  
tgaatttcgaagcttg
```

Obtained sequence, pCR II-TOPO-promoter of Bt407\_0422 in Bt 407,  
S1\_pUC19F:

---

```
ggatccaccttccttaattaacataaacaacaaaaacaaaatatatgaatatttgtttgaaaatgtttctcaatatgttcgagaataaaaaaaaa  
gccaatgtaaaagcattggccaagaccaaccctatccgtccggaactggctagcgtagccttcgaccttagctcctgtagtttgcgctc  
ctcatgtttccatcagtttgcctttctcgctgaacgagttgaacgatatgggagttctatgagaattaatttattctcttcaaagattacaacat  
ataaatcaaaatgtctacttatttagagatataaatacaaattttaatgcattgcttttcaaaataatttcaaaaagtagctgaatgttactttt  
ttgtgttatataataaaaaatgtataaacaacaaggatttcttcgggtatttatttaatttcattcaaattaactattcgaatatacttaactaa  
tgaatttcgaagcttg
```

Emboss analysis (<http://www.ebi.ac.uk/Tools/es/cgi-bin/jobresults.cgi/needle/needle-20091109-1341299280.html>, 9/11-2009) revealed no mutations.

## Appendix 2: Sequence analysis of promBt407\_1060-TOPO (9-11-2009)

Original sequence; pCR II-TOPO-promoter of Bt407\_1060 in Bt 407, forward direction:

```
ctgcagcccgagttaagagaaaaagtgattcaagctcttattggaattcatataaatagaaatatacgaactatataatgcgaaaataattc
accattgcttcttaatgcagtcgtgaattattttttattagaaaaatagaacaattttatagagtattgaattataaccgtccagacgggtata
gtaatatTTGGTTAagaggaaaatgaacaaaaaaaaagaataaaagatatTTTtagcacactattcgaaaggataggccgcaaagctta
gagtctacggtaatacatattggttactaagatcgtctggttgacattttgatgcaaccggacgggtctttttgttatataaatataaaaaa
taggaggggaactatgcaaaggatcc
```

Obtained sequence, pCR II-TOPO-promoter of Bt407\_1060 in Bt 407, S2\_pUC19R:

```
ctgcagcccgagttaagagaaaaagtgattcaagctcttattggaattcatataaatagaaatatacgaactatataatgcgaaaataattc
accattgcttcttaatgcagtcgtgaattattttttattagaaaaatagaacaattttatagagtattgaattataaccgtccagacgggtata
gtaatatTTGGTTAagaggaaaatgaacaaaaaaaaagaataaaagatatTTTtagcacactattcgaaaggataggccgcaagctta
gagtctacggtaatacatattggttactaagatcgtctggttgacattttgatgcaaccggacgggtctttttgttatataaatataaaaaa
taggaggggaactatgcaaaggatcc
```

Emboss analysis (<http://www.ebi.ac.uk/Tools/es/cgi-bin/jobresults.cgi/needle/needle-20100428-1516547298.html>, 28/4-2010) revealed a single mutation. Complementary, reverse sequence was analyzed.

Original sequence; pCR II-TOPO-promoter of Bt407\_1060 in Bt 407,  
complementary, reverse strand:

---

ggatcctttgcatagttccctcctatTTTTatattatataacaaaaaagaccgtccggtgcatcaaaaatgtgcaaccagacgatcttag  
taccaatatgtattaccgtagactctaagcttgcggcctatccttcgaatagtgctaaaaatatctttattctTTTTgttcattttcctct  
taaaccaaatattactataaccgtctggacggtataattcaatactctataaaattgttctatttttctaataaaaaaataattcacgactgcat  
taaagaagcaatggtgaattatttcgcattatatagttcgtatattctatttatatgaattaccaataagagcttgaatcactttttctttaactc  
gggctgcag

Obtained sequence, pCR II-TOPO-promoter of Bt407\_0422 in Bt 407,  
S1\_pUC19F:

---

ggatcctttgcatagttccctcctatTTTTatattatataacaaaaaagaccgtccggtgcatcaaaaatgtgcaaccagacgatcttag  
taaccaatatgtattaccgtagactctaagctctgcggcctatccttcgaatagtgctaaaaatatctttattctTTTTgttcattttcct  
cttaaaccaaatattactataaccgtctggacggtataattcaatactctataaaattgttctatttttctaataaaaaaataattcacgactgc  
attaaagaagcaatggtgaattatttcgcattatatagttcgtatattctatttatatgaattaccaataagagcttgaatcactttttctttaac  
tcgggctgcag

Emboss analysis (<http://www.ebi.ac.uk/Tools/es/cgi-bin/jobresults.cgi/needle/needle-20100428-1519535130.html>, 28/4-2010) revealed the same mutation on the complementary strain.

### Appendix 3: Sequence analysis of PCR product of promBt407\_1060 from Bt407 template (11-11-2009):

Original sequence; promBt407\_1060 from Bt 407, forward direction\*:

ctgcagcccgagttaagagaaaaagtgattcaagctcttattggttaattcatataaatagaaatatacgaactatataatgcgaaaataattc  
accattgcttcttaatgcagtcgtgaattatTTTTTattagaaaaatagaacaattttatagagtattgaattataaccgtccagacggtata  
gtaatatTTTggttaagaggaaaatgaaacaaaaaaaagaataaaagatatTTTtagcacactattcgaaaggataggccgcaaagctta  
gagtctacggtaatacatattggttactaagatcgtctggtgcacattTTTgatgcaaccggacggtctTTTTgttatataaatataaaaaa  
taggaggggaactatgcaaaggatcc

Obtained sequence; promBt407\_1060 from Bt 407, S2\_pUC19R:

atagagtattgaattataaccgtccagacggtatagtaatttgggttaagaggaaaatgaaacaaaaaaaagaataaaagatatTTTtag  
cacactattcgaaaggataggccgcaaagcttagagtctacggtaatacatattggttactaagatcgtctggtgcacattTTTgatgcaa  
ccggacggtctTTTTgttatataaatataaaaaata

Analysis of the marked region showed no sign of the mutation previously discovered.

Original sequence; promBt407\_1060 from Bt 407, complementary, reverse strand\*:

ggatcctttgcatagttccctctatTTTTtatattatataacaaaaaagaccgtccggtgcatcaaaaatgtgcaaccaagacgatcttag  
taaccaatatgtattaccgtagactctaagctttgcggcctatccttcgaatagtgctaaaaatatcttttattctTTTTgttcattttcct  
cttaaaccaaatattactataaccgtctggacggtataattcaatactctataaaattgttctatttttctaataaaaaaaataattcacgactgc  
attaaagaagcaatggtgaatttttcgcattatatagttcgtatattctatttatatgaattaccaataagagcttgaatcacttttctcttaac  
tcgggctgcag

Obtained sequence, promBt407\_1060 from Bt 407, S1\_pUC19F:

Gacgatcttagtaaccaatatgtattaccgtagactctaagctttgcgccctatccttcgaatagtgtgctaaaaatatctttattctttttt  
gtttcattttcctcttaaaccaaatattactataaccgtctggacggtataattcaataactctataaaattgttctatttttctaataaaaaataa  
ttcacgactgcattaaagaagcaatggt

Analysis of the marked region showed no sign of the mutation on the complementary strain.

\*Marked area show extent of generated sequence.

## Appendix 4: Sequence analysis of Bt407\_0422 - pHT304-pXyl construct (8/1-2010)

Original sequence Bt407\_0422 - pHT304-pXyl (forward):

TAAGGAAGGTGGATATTAATGAAA AAATATTGGCATAAGTTATCGTTCCTTCAAAGAACGTCTT  
ATTAACCGTATTAGTTATTTTGACACTTGTTGGAAGTATGGGTGCACTAAGTTTCAACATGTTTCA  
AAATAGTATGATGTCTTTATTTGAAAGGCAGTCTATTGAAACAGGAGAAATAGTATTGAAAAAAT  
TGGATGTAGAATTAGTTAGAGACATGGCGAAAGATCCTACAGCCGAAAAAGTGAAAAAGAAAA  
GTTAACAGAGAAATTAGATGAAGTGTCAAAGAATTGAAAAGTGTTGGACAAACGTATGTTACAG  
GAGCAAAGCCGAATGAAAAGCGAGAAATTACAACTCGTTGGTTTACTACAGAGTTAACAAAAGCG  
TTCCCTATAAAGCCGGGAGATTATTATGAACAACCCGCTCATTGGATGAAGGCATACGATAAAGT  
CATTGATACGAAAAAGGCCCAAATGACAGAAGTATATGAAGATGAAATAGGTTTCGTGGGTGACAA  
TATTAGAACCAATTACAGATGGAGAAAAACAATTATTGTTGCAATTATTGCGGCTGACTTAGATGCTT  
CCATTGTTCTTATTACAAAAGAGAAGTTTATGATACAGGGCTTACTCTTTATCATTATTTTCATTAGC  
GATTGCGACTATTACTCAATTCTTTATCGTGCGTCACTCATTAGCGCCATTAAAAGATTTACGAGA  
AGGGTTACGTAAAATTGGTGAAGGTGACTTGAGTATTAAATTAAGAAAGAACAGATGATATTG  
GAATTATTAATGTTTATTTCAATAACACGATTGAAAAGTTTAAAGGGATTATAGATAAAGTAAGG  
CAAACCTGCTGAGCAAGTTTCTTCTTCTCACAAGAATTGTCGGCGAGTACAGAGGAAAACAGCAT  
GGCAGTTCAAGAGATTGCAAGTTCTATTGAAGGTTTAAAGAGTAGGGGCACAGTCTCAAAAAACTT  
CAGTACAACAATGTTTAGG+GATTGTACATGGAATGGAAGATAAGGTAGAAGAGATAACTGGGGC  
TGCGAAACAAGTTGCGGTTGCTTCTGAAGGTATGGAGCAACATTCAATTGAAGGTAATGAAGTGA  
TTGGACAAATTATTAATCAAATGAGTTTAATTCAAAATGCAGTACAAGATTTATCTTCCATCATTT  
ATTCGTTAGAAAACAAGGTCAAAAGAAATTAGTGATATCGTAACTGTAATTACAGGTATTTCAAATC  
AAACAAATTTATTAGCTTTAAATGCTTCTATTGAGGCTTCACGTGCTGGAGCAGCGGGGAGAGGG  
TTTGCAGTTGTTGCTGATGAAGTACGAAAATTAGCAGAACAAACGGAAGCGTCTGCTAAAGATAT

AGCGAAATTAATTGGTGAAACACAAGCTGAGACAGAAGATGCTGTAGTTTCAATGCAAAAAGGTT  
 CAAAAGAAGTAGAACTGGAATTTCACTTGTTCAAAGTAGTGGGAATTTCTTTGAAAAAACTCC  
 AAATCAGCGCAATCTGTTACAGATCAAGTAAAAGCTGTTTCGGGTAATTTCGAGTGACATTTTACAA  
 AATAGCCAGAATATCGTTCAAGTCGTAAATGAATTATCACATATTGCAAACACTTATGCCAACAGC  
 AGTAGTAATGTTGAAGAAAGCATGAAAGAGCAAGAAATATCAGTACAAGATATTGCAGAATTAGC  
 GACTTCTTTAAGTTGGCTTTCTCAGGAATTACAAGAGCTTATTGGTGAGTTTAAAAGCGAATAAGG  
 AGAAAGTTCAATTCTAATCAAAGGGGATATCTCC

Original sequence Bt407\_0422 - pHT304-pXyl (reverse, complementary):

GGAGATATCCCCTTTGATTAGAATTGAACCTTTCTCCTTATTCGCTTTTAAACTCACCAATAAGCTCT  
 TGTAATTCCTGAGAAAGCCAACCTTAAAGAAGTCGCTAATTCTGCAATATCTTGACTGATATTTCT  
 TGCTCTTTCATGCTTCTTCAACATTACTACTGCTGTTGGCATAAGTGTTGCAATATGTGATAATT  
 CATTTACGACTTGAACGATATTCTGGCTATTTTGTAATAATGTCACTCGAATTACCCGAAACAGCTT  
 TTACTTGATCTGTAACAGATTGCGCTGATTGGAGAATTTTTCAAAAGAAATTCCCACTACTTTGAA  
 CAAGTGAAATTCAGTTTCTACTTCTTTTGAACCTTTTGCATTGAAACTACAGCATCTTCTGTCTC  
 AGCTTGTTGTTTACCAATTAATTTTCGCTATATCTTTAGCAGACGCTTCCGTTTGTCTGCTAATTTT  
 CGTACTTCATCAGCAACAACCTGCAACCCTCTCCCCGCTGCTCCAGCACGTGAAGCCTCAATAGAA  
 GCATTTAAAGCTAATAAATTTGTTTGATTGAAATACCTGTAATTACAGTTACGATATCACTAATT  
 TCTTTTGACCTTGTTTCTAACGAATAAATGATGGAAGATAAATCTTGACTGCATTTTGAATTA  
 CTCATTTGATTAATAATTTGTCCAATCACTTCATTACCTTCAATTGAATGTTGCTCCATACCTTCAG  
 AAGCAACCGCAACTTGTTTCGCAGCCCCAGTTATCTCTTCTACCTTATCTTCCATTCCATGTACAAT  
 CCTTAAACATTGTTGACTGAAGTTTTTTGAGACTGTGCCCTACTCTTAAACCTTCAATAGAACTT  
 GCAATCTCTTGAACAGCATGCTGTTTCTCTGTACTCGCCGACAATTCTTGTAAGAAGAAGAA  
 ACTTGCTCAGCAGTTTGCCTTACTTTATCTATAATCCCTTTAACTTTTCAATCGTGTTATTGAAAT  
 AAACATTAATAATTCCAATATCATCTGGTCTTTCTTTTAATTAATACTCAAGTCACCTTCACCAAT  
 TTTACGTAACCTTCTCGTAAATCTTTTAATGGCGCTAATGAGTGACGCACGATAAAGAATTGAGT  
 AATAGTCGCAATCGCTAATGAAATAATGATAAAGAGTAAGCCCTGTATCAAACTTCTCTTTTGT  
 AATAGGAACAATGGAAGCATCTAAGTCAGCCGCAATAATTGCAACAATATTGTTTTCTCCATCTGT  
 AATTGGTTCTAATATTGTCACCCACGAACCTATTTTCATCTTCATATACTTCTGTCAATTTGGGCCTTT  
 TTCGTATCAATGACTTTATCGTATGCCTTCATCCAATGAGCGGGTTGTTCAATAAATCTCCCGGCT  
 TTATAGGGAACGCTTTTGTAACTCTGTAGTCAAACCAACGAGTTGTAATTCTCGCTTTTCATTCTC  
 GCTTTGCTCCTGTAAACATACGTTTGTCCAACACTTTTCAATTCTTTTGACACTTCATCTAATTTCTC  
 TGTTAACTTTTCTTTTTTCACTTTTTTCGGCTGTAGGATCTTTCGCCATGTCTCTAACTAATTCTACAT  
 CCAATTTTTTCAATACTATTTCTCTGTTTCAATAGACTGCCTTCAAATAAAGACATCATACTATT

TTGAAACATGTTGAAACTTAGTGCACCCATACTTCCAACAAGTGTCAAAATAACTAATACGGTTAA  
TAAGACGTTCTTTTGAAGGAACGATAACTTATGCCAATATTTTTCATTAAATATCCACCTTCCTTA

Obtained sequence Bt407\_0422 with pHT304-pXyl\_forward:

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CCTTTATTATATCTAATGTGTTTCATAAAAACTAAAAAAATATTGAAAATACTGACGAGGTTATA  
TAAGATGAAAATAAGTTAGTTTGTTTAAACAACAACTAATAGGTGATGTACTTACTATATGAAAT  
AAAATGCATCTGTATTTGAATGAATTTATTTTAAAGGGGGAAATCMCATGGCTCAATCTAGATTAAGGAAGGTGGATATTAATGAAA

Obtained sequence Bt407\_0422 with 00330-Bt407\_0422-F2:

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GAAGTATATGAAGATGAAATAGGTTTCGTGGGTGACAATATTAGAACCAATTACAGATGGAGAAAA  
CAATATTGTTGCAATTATTGCGGCTGACTTAGATGCTTCCATTGTTCTTATTACAAAAGAGAAGTT  
TATGATACAGGGCTTACTCTTTATCATTATTTTCATTAGCGATTGCGACTTACTCAATTCTTTATC  
GTGCGTCACTCATTAGCGCCATTAAAAGATTTACGAGAAGGGTTACGTAAAATTGGTGAAGGTGA  
CTTGAGTATTAATTAAGAGAAAGACCAGATGATATTGGAATTATTAATGTTTATTTCATAACAC  
GATTGAAAAGTTTAAAGGGATTATAGATAAAGTAAGGCAACTGCTGAGCAAGTTTCTTCTTCTC  
ACAAGAATTGTCGGCGAGTACAGAGGAAAACAGCATGGCAGTTCAAGAGATTGCAAGTTCTATTG  
AAGGTTTAAAGAGTAGGGGCACAGTCTCAAAAACTTCAGTACAACAATGTTTAGGGATTGTACAT  
GGAATGGAAGATAAGGTAGAAGAGATAACTGGGGCTGCGAAACAAGTTGCGGTTGCTTCTGAAG  
GTATGGAGCAACATTCAATTGAAGGTAATGAAGTGATTGGACAAATTATTAATCAAATGAGTTTA  
ATTCAAATGCAGTACAAGATTTATCTTCCATCATTTATTCGTTAGAAACAAGGTCAAAAGAAATT  
AGTGATATCGTAACTGTAATTACAGGTATTTCAAATCAAACAAATTTATTAGCTTTAAATGCTTCT  
ATTGAGGCTTCACGTGCTGGAGCAGCGGGGAGAGGGTTTGCAGTTGTTGCTGATGAAGTACGAAA  
ATTAGCAGA



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Obtained sequence Bt407\_0422 with 00330-Bt407\_0422-R2:

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ATGACTTTATCGTATGCCTTCATCCAATGAGCGGGTTGTTTCATAATAATCTCCCGGCTTTATAGGG  
AACGCTTTTGTTAACTCTGTAGTCAAACCAACGAGTTGTAATTCTCGCTTTTCATTCGGCTTTGCTC  
CTGTAACATACGTTTGTCCAACACTTTTCAATTCTTTTGACACTTCATCTAATTTCTCTGTAACTT  
TTCTTTTTTCACTTTTTTCGGCTGTAGGATCTTTCGCCATGTCTCTAACTAATTCTACATCCAATTTTT  
TCAATACTATTTCTCCTGTTTCAATAGACTGCCTTTCAAATAAAGACATCATACTATTTTGAAACAT  
GTTGAAACTTAGTGCACCCATACTTCCAACAAGTGTCAAATAACTAATACGGTTAATAAGACGTT  
CTTTTGAAGGAACGATAACTTATGCCAATATTTTTCATTAATATCCACCTTCCTTAATCTAGATTG  
AGCCATGTGATTTCCCCCTTAAAAATAAATTCATTCAAATACAGATGCATTTTATTTTCATATAGTA  
AGTACATCACCTATTAGTTTGTGTTTAAACAACTAACTTATTTTCATCTTATATAACCTCGTCAG  
TATTTTCAATATTTTTTTTAGTTTTTATGAACACATTAGATATAATAAAGGGAAGATTTCGCTATGT  
ACTATGTTGATACTTAATTTAAAGATTAAACAAATGGAGTGGATGAAGTGGATATCGCTGATCAA  
ACCTTTGTCAAAAAAGTAAATCAAAAGTTATTATTAAGAAATCCTTAAATTCACCTATTTCAA  
GAGCAAAATTATCTGAAATGACTGGATTAAATAAATCAACTGTCTCATCACAGGTAAACACGTTA  
ATGAAAGAAAGTATGGTATTTGAAATAGTCAAGGACAATCAAGTGGCGGAAGAGACCTGTCATGC  
TTGTTTTAATAAAAAGCAGATACTCCGTTGGAATAGATGTTGGTGTGATATATAATGGCATTTACA  
GACCT

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Obtained sequence Bt407\_0422 with S2\_pUC19R:

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TGAACTTTCTCCTTATTCGCTTTTAAACTCACCAATAAGCTCTTGTAATTCCTGAGAAAGCCAACTT  
AAAGAAGTCGCTAATTCTGCAATATCTTGACTGATATTTCTTGCTCTTTCATGCTTCTTCAACAT  
TACTACTGCTGTTGGCATAAGTGTGTTGCAATATGTGATAATTCATTTACGACTTGAACGATATTCT  
GGCTATTTTGTAATAATGTCACTCGAATTACCCGAAACAGCTTTTACTTGATCTGTAACAGATTGCG  
CTGATTTGGAGACTTTTTCAAAGAAATCCCACTACTTTGAACAAGTGAATTCAGTTTCTACTT  
CTTTTGAACCTTTTTGCATTGAAACTACAGCATCTTCTGTCTCAGCTTGTGTTTACCAATTAATTT  
CGCTATATCTTTAGCAGACGCTTCCGTTTGTCTGCTAATTTTCGTACTTCATCAGCAACAACCTGCA  
AACCCTCTCCCCGCTGCTCCAGCACGTGAAGCCTCAATAGAAGCATTTAAAGCTAATAAATTTGTT  
TGATTTGAAATACCTGTAATTACAGTTACGATATCACTAATTTCTTTTGACCTTGTTTCTAACGAAT

AAATGATGGAAGATAAATCTTGTACTGCATTTTGAATTAACTCATTGATTAATAATTTGTCCAA  
TCACTTCATTACCTTCAATTGAATGTTGCTCCATACCTTCAGAAGCAACCGCAACTTGTTTCGCAG  
CCCCAGTTATCTCTTCTACCTTATCTTCCATTCCATGTACAATCCCTAAACATTGTTGTACTGAAGT  
TTTTTGAGACTGTGCCCCTACTCTTAAACCTTCAATAGAACTTGCAATCTCTTGAAGTGCCATGCTG  
TTTTCTCTGTACTCGCCGACAATTCTGTGAAAAGAAGAACTTGCTCAGCAGTTTGCCTTACTTT  
ATCTATAATCCCTTTAAACTTTTCAATCGTGTTATTGAAATAAACATTATAATTCCATATCATCTGG  
TCTTTCTTTTAATTTAATACTCAGTC

Analysis of the obtained sequences with the original sequence showed two mutations  
(marked red).

## Appendix 5: Sequence analysis after first mutagenesis (28/1-2010)

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Original sequence Bt407\_0422:

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tgaactttctccttattcgcttttaaactcaccaataagctcttgaattcctgagaaagccaactaaagaagtcgctaattctgcaatatctt  
gtactgatatttcttgctctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcattacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttggagatttttcaaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcgcat

Obtained sequence Bt407\_0422 colony 1:

---

Tgaactttctccttattcgcttttaaactcaccaataagctcttgaattcctgagaaagccaactaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgctctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcattacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttggagacttttcaa  
agaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcgcat

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#### Obtained sequence Bt407\_0422 colony 2:

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Tgaactttctcttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgcctttcatgcttcctcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagacgttttcaa  
agaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcatt

---

#### Obtained sequence Bt407\_0422 colony 3:

---

Tgaactttctcttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgcctttcatgcttcctcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagaggttttcaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaactacagcatcttctgtctcagcttggtt  
tcaccaattaattcgctatatcttttagcagacgcttccgtttg

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#### Obtained sequence Bt407\_0422 colony 4:

---

tgaactttctcttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatctt  
gtactgatatttcttgcctttcatgcttcctcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagaggttttcaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaac

---

#### Obtained sequence Bt407\_0422 colony 5:

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tgaactttctcttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatctt  
gtactgatatttcttgcctttcatgcttcctcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagaggttttcaa  
agaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaa

Obtained sequence Bt407\_0422 colony 6:

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Tgaactttctccttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgctctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttggagatttttcaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaactacagcatcttctgtctcagcttggtt  
tcaccaattaattcgctatatctttagcagacgcttcc

Colony 3, 4 and 6 did not contain the original mutation.

## **Appendix 6: Sequence analysis after second mutagenesis (3/2-2010)**

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Original sequence Bt407\_0422:

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Tgaactttctccttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaactaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgctctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttggagatttttcaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcatt

---

Obtained sequence Bt407\_0422 colony 1:

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Not generated, error done by the DNA sequence lab.

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Obtained sequence Bt407\_0422 colony 2:

---

Tgaactttctccttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaacttaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgcctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagatttttcaaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaactaca

---

Obtained sequence Bt407\_0422 colony 3:

---

Tgaactttctccttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaacttaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgcctttcatgcttcttcaacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaac  
gatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagatttttcaaa  
gaaattcccactactttgaacaagtgaattccagtttctacttctttgaaccttttgcattgaaactac

Obtained sequence Bt407\_0422 colony 4:

---

Tgaactttctcttattcgcttttaaactcaccaataagctcttgtaattcctgagaaagccaacttaaagaagtcgctaattctgcaatatct  
tgtactgatatttcttgctctttcatgcttcttcmacattactactgctgttggcataagtgttgcaatatgtgataattcatttacgacttgaa  
cgatattctggctattttgtaaaatgtcactcgaattacccgaaacagcttttacttgatctgtaacagattgcgctgatttgagatttttcaa  
agaaat

## **Appendix 7: Protein sequence of Bt407\_0422 and Bt407\_1060 in Bt 407**

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Bt407\_0422:

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MKKYWHKLSFLQKNVLLTVLVILTLVGSMGALSFNMFQNSMMSLFRQSIETGEIVLKKLDVELVRD  
MAKDPTAEKVKKEKLTEKLDEVSKELKSVGQTYVTGAKPNEKRELQLVGLTTELTKAFPIKPGDYEE  
QPAHWMKAYDKVIDTKKAQMTEVYEDEIGSWVTILEPITDGENNIVAIIAADLDASIVPITKEKFMIQG  
LLFIIISLAITITQFFIVRHSLAPLKDREGLRKIGEGDLSIKLKERPDDIGIINVYFNNTIEKFKGIIDKVR  
QTAEQVSSSSQELSASTEENSMAVQEIASIEGLRVGAQSQKTSVQQCLGIVHGMEDKVEEITGAAKQV  
AVASEGMEQHSIEGNEVIGQIINQMSLIQNAVQDLSSIIYSLETRSKEISDIVTVITGISNQTNLLALNASIE  
ASRAGAAGRGAFAVVADEVVRKLAEQTEASAKDIAKLIGETQAETEDAVVSMQKGSKEVETGISLVQSSG  
NFFEKISKSAQSVTDQVKAVSGNSSDILQNSQNIVQVVNELSHIANTYANSSSNVEESMKEQEISVQDIA  
ELATSLSWLSQELQELIGEFKSE

Bt407\_1060:

MKSTKRHISMVSLVFLVLFVSLNVWAPVIAAVMKSPVDEINISRTDGTTSPEYQASDGMKVEVKWS  
AKEKIKSGDQFTIDMPKEFRKDLNMMSFPLKDAEGKTVGTCEMCKGLLTCTMGNYVEGKNNIKGSLF  
VEFYFGLEAYDGVKEIPLEFNVDGQIVNKEVNVSNNTTERPKPQPNTDNLLKWGSYNQEDPSIADWL  
VYNATGTEMQDLKLTDTLGPHELITDSVVL EEAVFEDGYAPTNIKPADLSNIKINATKTGFTIEFPDSSK  
GYILRYKTKVTNPAAPHKNTVKLEGKNIKTEEKVGQVVFVSGGGSGSGDNNPPSIEKNIVDENGKLV  
ENEQLTQMDQVIQYQVGTHIPNDPPKYTSMVISDDLEDVLEVL EAKVYDQNGQDITSKGTNLIDKQRS  
EVTFTFGESFDYKSYEDQIINLSIKAKIKNNADLSSYVDKKIPNKAELHFDDKTLTSKEVTITPPEAPKDG  
TVSLHKIDSENPNKGLKAAEFVNRNSANEVVAKLKTDEKGFSVPQTLAPGTYKVYETVAPEGYQKLT  
PVEVTLQAGETKTIEIKNTMQKGQIEVKKIDSENGEKPLANAEFDIVKDGVVVEHIVTDKDGKAISKPL  
APGKYILKETKAPEGYQLKETEFVNVVTGDGIFPIQVENAMVDKGNIEITKVDKENGAVLAGVEFEVQ  
DEKDGVVVRKVVTDKGKANVSDLSVGKYKLVETKSLPGYKNLAEPVLFEITKGMTKALVIK VENEQL  
DKGSVEITKIDKDSQKVLEGVVFEVQDEQGKVVEVKTDKDGKAKISDLSVGKYKLVETKSLPGYKK  
LTEPVSFEITKGMTTVLSLK VENEQLDKGSVEITKVDKDSKKALKGVVFEVQDEAGTVVKEVKTDKD  
GKAKISDLLVGKYKLV EKESLPGYKKLTDPVLFEIKKGMTKVL SLKIENEMVDTGNVEITKIDKDNKA  
PLAGVT FIVQDEKGNVTKVTTDKDGKANVSDLPVGKYELVEVESLPGYKKLEKPV SFIEKKGMTEV  
LSLKVENEMVDTGNVEITKIDKDSKAPLENVVFEVRDLKGKVVAKVTTDKGKANVSDLP IGYELV  
EVETPAGYKPLEKPISFEIEKGRVTALKLTVENELVDTGNVEITKVDKENKDALADAVFEIQDEAGQVV  
AKITTDKKGQAQVTNLSVSTYKLV EVKAPKGYKQLVDPITFQIEKGMTKSLALTVENEMLDKGNVEV  
TKVDKDSQKVLEGVVFEVQDDKGVVTEVTTDKGKANVSDLSVGKYKLVETKSLPGYKKLT EPVS  
FEIKKGMTKVL SLK VENEQLDKGSVEITKVDKESGAVLAGVT FEVQDEKDKVVTKVKTDKGKANVS  
DLSVGKYKLV EVESLPGYKKLAKPV SFIEKKGMTEVLSLK VENEQLDKGSVEITKVDKDSQKALEGVT  
FEVQDEKGVVTKVTTDKGKV KISDLSVGSYKLV EENLPGYKKLT EPVSFEIKKGMTEVLSLKVEN  
EQLDKGSVEITKVDKDSQKVLEGVVFEVQDEQGKVVEVKTDKNGKAKISDLSVGKYKLV EKESLPG  
YKKLT EPVSFEIKKGMTEVLSLK IENEMVDTGNVEITKIDKDNKAPLAGVVFEVQDDKGVVTKVTT  
DKAGKATVSDLSVGKYKLV EVDLSLPGYKKLEKPV PFIEKKGMTKSLTFTVENEMVDTGNVEITKIDK  
DSKARLENVVFEVRDSKGKVVAKVTTDKGKANVSDLSIGKYELVEVETPAGYKPLEKPV SFIEKGR  
VTALQLTVENELVDTGNIEITKVDKENKDALADAVFEIQDAAGQVVAKITTDKKGQAQVTNLSVGT  
YKLV EVKAPKGYKQLVDPITFQIEKGMTKSLALTVENEMLDKGNVEVTKVDKDSQKVLEGVVFEVQD  
EQGKVVEVTTDKGKANVSDLSVGKYKLVETKSLPGYKKLT EPVSFEIKKGMTKVL SLK VENEQLD  
KGSVEITKMAAESKEVLSGAVFEVHDEKGVVVKVTTDKGGKAKIADLSVGN YTLVEVEAPKGYEK  
LTNPIPF EITNGMINAVQLEVLNKLNLHLAPPGPETPDPEKPGTPDPEKPGTPDPEKPGTPNPEKPGTPDP  
EKPGTPNPEKPGTPDPEKPGTPNPEKPGTPDPEKPGTPNPEKPGTPNPEKSEKELPKTGQKMPVEPYMG  
ALLVMMSFGLFVLGRKQQR

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